

**MANAGEMENT OF POSTHARVEST DECAY OF TOMATO FRUIT WITH
INORGANIC SALTS AND NATURAL PRODUCTS**

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ABSTRACT

Gray mold caused by *Botrytis cinerea* and soft rot caused by *Pectobacterium carotovorum* are two of the most devastating pre- and postharvest diseases of tomato worldwide. A survey of fresh-market tomato fruit was conducted to determine the fungal and bacterial pathogens most commonly associated with postharvest disease on Oahu, Hawaii. The survey confirmed that gray mold and soft rot were the most common postharvest diseases of tomato in Hawaii. Pathogenicity tests revealed that 33 of 99 fungal isolates and 10 of 17 bacterial isolates were pathogenic on tomato varieties known as common market, cherry, and grape tomato. Based on fruit assays one fungal isolate, *Botrytis cinerea* (B03) and one bacterial strain, *Pectobacterium carotovorum* (BA17), were selected because both produced consistent symptoms and were highly virulent. As consumer-acceptable options for postharvest disease management are inadequate for gray mold and soft rot, experiments were designed to evaluate possible control measures for these two diseases. Effects of eleven natural products on spore germination and mycelial growth of *Botrytis cinerea* (B03) were evaluated using multi-well microplates and inhibitory assays, respectively. *Capsicum chinense* cv. Datil, *C. annuum* cv. Carnival, and an Agrichem Proprietary Formulation (APF) completely inhibited fungal germination at all evaluation times. Treatments with 40% plant extracts increased the generation of intercellular reactive oxygen species and the plasma membranes of fungal conidia were damaged. Fungal spores exposed to 40% plant extracts of *C. chinense* or *C. annuum* or 1 ml/L APF showed distinct signs of deterioration, deformation and condensed cytoplasm as observed using transmission electron microscopy. *Capsicum chinense* and *C. annuum* applications at 60% completely inhibited lesion development on tomato fruit following immersion for 10 min. An inorganic salt, potassium tetraborate tetrahydrate ($B_4K_2O_7 \cdot 4H_2O$) (PTB), was evaluated for

effects on the growth of *P. carotovorum* using strain BA17. Complete inhibition of bacterial colony development was achieved by treatment with PTB at 100 mM both at pH 9.2 and after adjustment to pH 7.0. Bactericidal activity was quantified and validated by counting fluorescently-labeled live and dead bacterial cells using flow cytometry, and reconfirmed using qPCR with high-affinity photoreactive DNA binding dye propidium monoazide (PMA). The results of flow cytometry, qPCR, and culturing confirmed that bacterial cells were killed following exposure to PTB at 100 mM. Bacterial cell membranes were damaged following a 5-min treatment and extrusion of cytoplasmic material from bacterial cells was observed using electronic transmission microscopy. Soft rot incidence on inoculated tomato fruit was reduced by dipping infected fruit in PTB at 100 mM for 5 min and no lesions developed following a 10-min treatment. PTB and APF were assessed as a preharvest spray in the greenhouse for reducing gray mold of tomato fruit caused *Botrytis cinerea*. PTB and APF were applied at three ripening stages: turning, light red, and pink. Both compounds reduced the disease severity of gray mold in greenhouse tomatoes when applied at the turning stage when fruit were stored either at 4 or 25°C. In this study, recombinase polymerase amplification (RPA) was used for specific and rapid detection of *Pectobacterium* using lateral flow strips. The assay also included tissue from host plants (tomato and potato) as an internal control to enhance the reliability and accuracy of the assay. The assay readily distinguished *Pectobacterium* from other bacterial genera and proved to be an efficient assay for identification of the target pathogen in plant tissues. In summary, APF and PTB show promise for reducing gray mold as preharvest applications in the greenhouse tomato. In contrast, PTB is an effective alternative to other bactericides and antibiotics for controlling soft rot disease of tomato as a postharvest practical application.

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LIST OF ABBREVIATIONS

APF	Agrichem proprietary formulation
CFU	Colony forming units
Ct	Cycle threshold
DCFH-DA	2,7-Dichlorofluorescein diacetate
EPA	United States Environmental Protection Agency
FDA	U.S. Food and Drug Administration
Fg	Femtogram
HCL	Hydrochloric acid
KOH	Potassium hydroxide
L	Liter
mL	Milliliter
mM	Millimolar
NaCl	Sodium chloride
Ng	Nanogram
OF	Oxidative-fermentative metabolism
PCR	Polymerase chain reaction
PI	Propidium iodide
PMA	Propidium monoazide
PTB	Potassium tetraborate tetrahydrate
qPCR	Quantitative polymerase chain reaction
ROS	Reactive Oxygen Species
μl	Microliter
USDA	United States Department of Agriculture

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Global importance of food and vegetable crops

Vegetables play a key role in the global economy and are grown in at least 200 different countries. Fruits and vegetables are major sources of vitamins and minerals in the human diet. Kays (2011) estimates that 402 vegetable crops planted internationally are destined for fresh market and few of them are used for processing. Most of the vegetable crops are cultivated in Asia, principally in China, which produces more than 50% of world's vegetables. The demand for vegetables has increased in different parts of the world as people have become increasingly aware of their nutritional benefits. Tomato is not an exception and has numerous health benefits.

The tomato

Tomato (*Solanum lycopersicum* Mill) is one of the most important vegetables produced globally, comprising approximately 14% (881 million tons) of world vegetable production (USDA-ERS, 2016). In addition, tomato is one of the most universally consumed foods in the world because of its variety of uses in cooking. The total of U.S tomato production is highest among all vegetable crops and the United States is the second largest producer of tomato in the world. More than \$2 billion in annual farm cash receipts comes from fresh and processed tomato (USDA-ERS, 2016). Another feature of tomato is its high yield over a short production period. Therefore, it is a commercially significant vegetable crop and its importance is increasing. Florida and California are largest fresh market tomato-producing states in the U.S. providing around 470,000 tons in 2015 (Statista, 2015). The demand for tomato in the U.S. is high throughout the year (USDA-ERS, 2016). Another factor that increases the value of tomato is its nutrition. Tomato fruit contain vitamins and minerals essential for human health such as vitamins

B and C as well as iron and phosphorus. Recognition of its multiple properties by consumers has increased the economic importance of tomato products, either in fresh or processed form (Naika et al., 2005). Postharvest diseases, however, cause considerable losses.

The principle causes of postharvest losses on tomato

Postharvest handling and storage conditions reduce the quality and quantity of products. Many preharvest factors affect the development of postharvest diseases including rainfall, temperature, irrigation methods, fungicide applications, plant density, selection of cultivars, etc. (Sablani et al., 2006). Mechanical injury and subsequent disease development are the most common causes of losses followed by poor storage condition. In addition, postharvest losses are increased through improper handling, transport, chilling injury, fluid loss, or delayed sales due to overproduction. Many countries lack appropriate handling, storage, packaging, and transport mechanisms, resulting in additional of postharvest losses. Storage at improper temperatures favors infection by several types of fungal and bacterial pathogens (Coates and Johnson, 1997).

Types of postharvest losses

Quantitative and qualitative losses are the common classifications in the postharvest system. Physical losses (weight, decay, and poor appearance) contribute to a decrease in product value (Khan and Jan, 2007). Postharvest losses affect marketing price and potential profits from commodities and products (Kader, 2004). Qualitative losses such as color appearance, taste, and decay are major considerations for consumers.

Global summary of postharvest losses

A summary of primary and secondary causes for postharvest losses, ranging from biological to physical, is shown in (Table 1.1). Estimates of the percentages of losses by region and country are shown in (Table 1.2). Several African countries, such as Benin, Ghana, and

Rwanda, show lower average losses, possibly due to direct marketing by farmers or possibly greater acceptance of damaged fruits and vegetables by consumers in those countries as compared to Europe and the Americas.

Table 1.1. Classification of postharvest vegetable losses and their major causes (Marsh et al., 2001).

Primary causes	Secondary causes
Biological	Preharvest management
Microbiological	Harvesting methods and handling
Chemical	Storage type
Biochemical	Transport mode, type, and availability
Physiological	Refrigeration facilities
Mechanical	Drying equipment
Environmental	Marketing and processing system
Pathological	Legal standard in place
Physical	Tool maintenance

Table 1.2. Regional postharvest losses of fruit and vegetables^a

Region	Country	Loss %
Africa	Ethiopia	25-35
	Zimbabwe	35-45
	Nigeria	20-30
	Benin	14-18
	Ghana	13-17
	Rwanda	10-21
	Mean	20-28
Asia	China	15-35
	Oman	3-19
	Indonesia	20-50
	Iran	>35
	Korea	20-50
	Philippines	27-42
	Sri Lanka	16-41
	Thailand	17-35
	Vietnam	20-25
	China	20-25
	India	25-40
	Mean	20-36
Europe	UK	25
	UK retail out Grades	25-40
	Mean	25-33
Americas	USA	23-25
	Brazil	20
	Mean	22-23
Global	Mean	31-38

^a Data compiled by (Munhuwiyi, 2012)

The most common fungal and bacterial postharvest diseases of tomato

Fungal and bacterial pathogens cause postharvest diseases in fruit and vegetables. Some diseases are severe when appropriate conditions, such as mechanical injury causing breaks of the cuticle, high temperature and moisture, favor infection after harvest whereas other postharvest diseases are due to infections that occurred prior to harvest. Fruits and vegetables are often infected through surface injuries occurring during or after harvest, permitting colonization by various fungi, including *Rhizopus* sp. (Rhizopus rot), *Alternaria* sp. and *Stemphyllium* sp. (black mold), *Fusarium* sp. (Fusarium rot), *Phoma* sp. (Phoma rot), *Colletotrichum* spp. (anthracnose), *Botrytis* spp. (gray mold), *Cladosporium* sp. (Cladosporium rot), *Geotrichum* sp. (sour rot) and bacteria, including *Pectobacterium carotovorum* (bacterial soft rot), and *Lactobacillus* spp. (sour rot) (Bartz et al., 2009).

Chemical control of postharvest diseases

Postharvest diseases are controlled by different fungicides and bactericides or biological control that prevents the development of pathogens on the surface of the infected fruits. The most frequently used chemicals are sodium o-phenyl phenate, dichloran, biphenyl, amino butane, thiabendazole, benomyl, thiophonate- methyl, imazalil, chlorothalonil, triforine, captan, borax, vinclozolin, soda ash, and iprodione (Agrios, 2005b). Most of these pesticides require contact with the fruit surface to kill target organisms.

Appropriate timing and application strategies are critical for disease prevention. Postharvest application prevents infection through wounds and decreases development of infection during storage. Therefore, understanding the mode and time of infection is essential before selecting an appropriate strategy for management specific diseases.

Previous studies showed weekly field application of fungicides to papaya foliage reduced the development anthracnose caused by *Colletotrichum gloeosporioides* and *Alternaria alternata* (Alvarez et al., 1977; Alvarez and Nishijima, 1987). (Prusky et al., 1983) reported that application of fungicide sprays after mango fruit set reduced latent infections the incidence of black rot caused by *A. alternata* during storage. In addition, mancozeb field sprays reduced incidence of *Rhizopus* soft rot in postharvest evaluations (Nishijima et al., 1990). Some studies found that the antifungal compound, imazalil, was effective in decreasing *Colletotrichum* sp. on banana (Khan et al., 2001) and *Alternaria* sp. on tomato and bell peppers (Spalding, 1980; Spalding and King, 1981). Other fungicides such as Ridomil, Fosetyl Al, and Etaconazole were effective against various fungi of fruit and vegetables (Barkai-Golan, 2001). According to (Wang and Morris, 1992), a postharvest application of guazatine and borax decreased the incidence of fungal diseases of tomato at the mature green stage. Postharvest treatment of fresh market tomato with fungicidal wax including phenyl phenol was effective in reducing decay (Hall, 1989). However, chemical control is unsuccessful for soft rot caused by *Pectobacterium* sp. (formerly *Erwinia* sp.) (Farrar et al., 2009). Chlorine (8% CaCl_2) controls tomato soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* (Gomes et al., 2005). Even though chemical control has been essential for controlling postharvest diseases, global concerns have increased and there is a trend toward eliminating chemical applications because of potential side effects and hazards to human health as well as potential development of pathogens resistant to fungicides (Norman, 1988).

Recently, glacial acetic acid (used either as liquid or vapor) was effective in reducing *Alternaria alternata* and *Botrytis cinerea* on tomato. Also, the treatment of infected tomato with

40 µm/L acetic acid vapor extended the storage life of fruit up to 16 days as its concentration was increased (Alawlaqi and Alharbi, 2014).

Biological control

Chemical control of postharvest diseases has been the primary approach to decreasing fruit and vegetable losses since the 1960s. Although effective, international concerns for public health have resulted in introduction new regulations and policies to limit the use of chemical pesticides over the past decades. This shift to alternative methods has accelerated for several reasons, including the potential side effects of pesticides and human safety, environmental pollution, the evolution of pathogenic strains resistant to different pesticides, and the concern for residual pesticides on the fruit. Currently, consumers are concern about the quality of food that is free from microbial growth and toxins. Previous studies indicated that the effectiveness of *Bacillus amyloliquefaciens* (strain 5PVB) in reducing gray mold caused by *Botrytis cinerea* in fresh market tomato less than 20 °C in a storage (Mari et al., 1996). Some research showed that antifungal compounds in *Saccharomyces* was effective in reducing the growth of *Colletotrichum coccodes* spores and prevent the decay development in tomato fruit (Jones and Prusky, 2002). In addition, previous research documented that the activity of the yeast, *Pichia guilliermondii* was sufficiently successful in reducing the fungal growth caused by *Rhizopus stolonifer*, *Alternaria solani*, and *Botrytis cinerea* on tomato fruit (Zhao et al., 2008). *Trichoderma harzianum* has been utilized as biological control agent against fungal postharvest diseases on tomato fruit (El-Katatny and Emam, 2012). Bacteriophage (pp1) was active as a biological agent against bacterial soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* (Lim et al., 2013).

Plant extracts used as antimicrobial agents for postharvest diseases of tomato

Many applications of synthetic chemical products have been used to control fungal and bacterial postharvest diseases. Plant extracts may protect fruit from spoilage by inhibiting microbial growth. Antimicrobial characteristics of various extracts have been described for their uses in plant protection. Chitosan as natural compound reduced the growth of pathogenic fungi *in vitro* such as *Alternaria alternata*, *Botrytis cinerea*, *Colletotrichum gloeosporioides* and *Rhizopus stolonifer* (Bautista-Baños et al., 2006). In addition, some plant extracts, such as *Azadirachta indica* seed, *Garcinia kola*, *Zingiber officinale*, *Piper guineense* seed and *Myristica fragrans*, essential oils of *Coriandrum sativum*, *Rosmarinus officinalis* and *Eucalyptus globulus* are used at different concentrations to reduce bacterial soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* (Emma et al., 2013). Other studies show that the essential oils or vapors obtained from plant materials were able to control postharvest diseases in tomato and extend their shelf life. Ethanolic extracts of tarragon, rosemary, thyme, and the essential oil of oregano are powerful inhibitors of *Penicillium* spp., *Rhizopus* spp., *Fusarium* spp., *Aspergillus niger*, and *A. flavus* (Ibrahim and Al-Ebady, 2014).

Antimicrobial activity of *Waltheria indica*

Waltheria indica L. (Sleepy morning) is a woody perennial plant belongs to Sterculiaceae family and it is widespread in subtropical and tropical regions (Saunders, 2007). This plant has been utilized in traditional medicine in Hawaii (Abbott and Shimazu, 1985), South Africa (Mathabe et al., 2006), South America (Leonard and Ac, 1998) and India (Bapuji and Ratnam, 2009). *Waltheria indica* is considered as an indigenous plant in the Hawaii Island, and it is used as medicine for the treatment sore throat, cough, inflammation, and asthma. In Hawaii, *W. indica*

is most widely used as a medicinal plant and it is locally available as anti- inflammatory drug (Saunders, 2007).

Waltheria indica contains many chemical compounds including alkaloids, flavonoids, sterols, saponins, terpenes, cardiac glycosides, anthraquinones, and carbohydrates (Rao et al., 2005; Maheswara et al., 2006). The antifungal activity of *W. indica* has been demonstrated in several studies (Garba et al., 2012). A flavonoid compound isolated from *W. indica* show inhibition of *Candida albicans*, which is responsible for diarrhea in humans (Ragasa et al., 1997). (Zongo et al., 2013) claimed that the antifungal activity of *W. indica* can be supported by the permeability of fungus membrane.

Recently, many studies relate the antibacterial activity of aqueous and ethanol extracts from *W. indica* have been documented (Garba et al., 2012; Mongalo et al., 2012). *Waltheria indica* has not only antifungal and antibacterial activity but also antiviral activity. Some studies showed that water and methanol shoot extracts of *W. indica* are able to inhibit HIV 1(strain IIIb) which is responsible for AIDS disease (Maregesi et al., 2010).

Bioactive compounds in *Capsicum* pepper

The diploid self pollinating hot *Capsicum* pepper is a member of the family Solanaceae, is closely related to tomato, potato, eggplant and tobacco. Pepper is one of the most common and widely grown spice worldwide (Bosland, 1996). The genus *Capsicum* comprises many sweet or hot cultivars but *C. chinense*, *C. annuum*, *C. frutescens*, *C. baccatum*, and *C. pubescens* are the most common cultivated species. They contain different concentrations of capsaicin, dihydrocapsaicin, and nondihydrocapsaicin that determine the level of pungency (Karjewska and Powers, 1988; Antonious, 2017). In addition, *Capsicum* cultivars contain a variety of phytochemical compounds, including carotenoid and phenolic content that have antioxidant

properties (Deepa et al., 2007). The antifungal and antibacterial properties of capsaicin have been described in several studies (De Lucca et al., 2006; Dorantes et al., 2008; Omolo et al., 2014; Gayathri et al., 2016).

Inorganic salts potassium tetraborate tetrahydrate PTB

Many organic and inorganic salts have been used in food preservation and for controlling plant diseases. Sodium thiosulfate, aluminum chloride, and sodium benzoate have been used in postharvest applications to control soft rot disease of potato (Yaganza et al., 2014). Smilanick et al., (1999) reported that since the 1930s bicarbonate and carbonate salts have been reported to control diseases such as citrus mold caused by *Penicillium* sp., decays of *melon* caused by *Fusarium* sp. and *Rhizopus* sp. (Aharoni et al., 1997) and powdery mildew of cucurbit plants caused by *Sphaerotheca fuliginea* (Ziv and Zitter, 1992). Potassium tetraborate tetrahydrate ($K_2B_4O_7 \cdot 4H_2O$) or PTB is an inorganic salt resulting from the controlled reaction of potassium hydroxide, water, and boric acid. PTB has higher solubility in water than borax. However, Borax is banned in the USA for use as a food additive or for fruit and vegetables coatings (FDA, 1998). Borate has been reported to be an effective antimicrobial agent against several enteric bacteria and it is suitable for use as an ophthalmic pharmaceutical agent (Houlsby et al., 1986). Potassium tetraborate also has been reported to control or documented as a control for several postharvest diseases such as gray mold of table grape caused *Botrytis cinerea* (Qin et al., 2010); mango rot caused by *Colletotrichum gloeosporioides* (Shi et al., 2011) and brown peach rot caused by *Monilinia laxa* (Thomidis and Exadaktylou, 2010).

The effect of potassium tetraborate PTB on human health

Potassium tetraborate PTB ($K_2B_4O_7 \cdot 4H_2O$) is a white crystalline product resulting from a controlled reaction of potassium hydroxide, water and boric acid. “PTB is one of the boron

compounds which is most commonly used in many areas of industry although very limited information is available concerning its toxicity” (Çelikezen et al., 2014). PTB has been used as disinfectant, detergent, and for treatment of contact lenses worldwide. In addition, approximately 189 pesticides registered in the United States contain boric acid and its sodium salts as an active ingredient (EPA, 1993). Consumed food is the primary source of boron acquired by the human body. Some fruits and vegetables, such as legumes and nuts contain high levels of boron, whereas others have low levels. Humans may acquire boron through beverages, water and through skin contact (Nielsen, 1997). Boron does not accumulate in human tissues being rapidly excreted in urine as boric acid (H_3BO_3) (Sutherland et al., 1998). The average quantity of boron ingested daily by human beings differs with gender and age. It was documented that the average of daily boron intake by in adult males was 1.52 mg/day (Anderson et al., 1994).

Currently, sufficient knowledge and information about toxic levels of potassium tetraborate salts is unavailable. However, in a recent study, Çelikezen et al (2014) demonstrated that no concentrations of PTB (0-1280 µg/ml) were genotoxic when tested on human blood cell cultures. In addition, the total antioxidant capacity of human peripheral blood lymphocytes increased at low concentrations of PTB (1.25- 5 µg/ml) and no change in the total oxidative status of blood lymphocytes is observed.

Food safety issues

Increased incidence of food borne illnesses is associated with consumption of fruit and vegetables. These illnesses have been connected with several groups of microorganisms such as bacteria, viruses, and fungi (Tauxe et al., 1997). Some studies have shown that salmonellas are associated with the most common food- borne infections and approximately 1.2 million U.S illnesses annually have been traced to food (Scallan et al., 2011). In addition, world trade is

affected by food safety. For some countries, agricultural exports play an important role in the economy and contaminated imports consumed by the local population are hazardous to public health. Fresh products can be contaminated with pathogenic and nonpathogenic microorganisms at various stages of plant growth, development harvesting, handling, processing, packaging, and consumption. The most important organisms associated with food safety issues and human diseases are bacteria such as *Salmonella*, *Escherichia coli*, and *Shigella* and fungal mycotoxins (Hedberg et al., 1999). More attention must be given to safety issues of fresh produce and reducing worldwide risks.

Wash water of fresh tomato is the main source of bacterial and fungal contamination by bacteria and fungi such as *Bacillus cereus*, *Lactobacillus spp.*, *Staphylococcus aureus*, *Penicillium spp.*, *Aspergillus niger* (Ofor et al., 2009). Other studies on postharvest tomato have shown that *Trichothecium roseum* pose a risk to human health because this fungus is known as a producer of mycotoxins (Dal Bello, 2008).

The impact of fungal mycotoxins

Mycotoxins are low molecular- weight, secondary metabolites, produced by filamentous fungi that are toxic to animals and humans (Bennett and Klich, 2003). The most important categories of mycotoxins are trichothecenes, fumonisins, aflatoxins, and ochratoxins. These compounds can cause health issues such as abdominal pain, cancer in different target organs, and even death (Moss, 2002). The Food and Agriculture Organization (Egmond, 2002) indicate that 25% of the global commodities are affected by mycotoxins every year.

Fungal postharvest diseases of tomato not only cause economic losses due to blemishing and decay but also increases the risk of contamination by fungal mycotoxins such as aflatoxin, ochratoxin, sterigmatocystin, patulin, penicillic acid, citrinin, zearalenone, alternariol

monomythylether (AME), Alternariol (AOH) and trichothecenes. Mycotoxins have received considerable public attention due to potential risk to human and animal health. Fungal mycotoxins cause some level of acute toxicity when ingested in high amounts (Motta and Soares, 2000). The most important fungal genera that produce mycotoxins are *Aspergillus*, *Alternaria*, *Penicillium*, *Fusarium*, *Claviceps*, and *Trichothecium* (Bennett and Klich, 2003). The risk to the consumers occurs when these mycotoxins contaminate food products and as eaten.

Reactive oxygen species (ROS)

Reactive Oxygen Species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide ($\text{O}_2^{\cdot-}$) are a byproduct of an aerobic organism lifestyle, generated primarily by reduction of oxygen during respiration or oxidoreductase enzymes and metal catalyzed oxidation (Halliwell and Gutteridge, 2015). Cells have several defense mechanisms to balance production and removal of ROS. An imbalance of ROS often leads to oxidative stress and eventually the cell death (Held, 2012). Within cells, ROS groups can react rapidly with DNA, proteins, and lipids leading to DNA damage, lipid peroxidation, and protein oxidations and cell death (Beckman and Ames, 1998). Reactive oxygen species have been studied to be one of the potential mechanisms for damage and killing fungal spores and bacterial cells exposed to several compounds. Some studies showed that high ROS accumulation was the main reason of oxidative damage of *Botrytis cinerea* spores upon exposed at 24 mg/L selenium result in lower spores germination (Wu et al., 2016). Other studies have been shown that *Penicillium expansum* spores exposed to the potassium borate generates ROS and leads to oxidative stress and prevent the germination of fungal spores (Qin et al., 2007).

Flow cytometry

Flow cytometry is accomplished through the use of a sophisticated instrument that is able to detect the optical and fluorescence features of a single cell or other cellular components such as nuclei or chromosomes in a suspension stream when exposed to the light source (Wilkerson, 2012). Prepared cell suspension is essential for flow cytometric analysis. Fluorescent dyes can bind to the antigens or compounds of interest. Cell suspensions are exposed to a laser beam upon passing through the fluidics system. The laser beam is either scattered or absorbed when it hits a cell or organelles. Absorbed particular wavelength can be re-emitted as fluorescence if the cell contains a labeled fluorescent substance (Adan et al., 2017). The physical characteristics of the particles can be measured by light scattering, whereas fluorescence emission can be measured from a fluorescent dye that binds the cell or cellular components. Flow cytometry is used in a variety of laboratory applications because it is rapid, accurately enumerates cells and when coupled with specific reagents it distinguishes between live and dead cells. Cell viability can be determined through traditional culture-based tests, but culturing methods are time-consuming and not appropriate for nonculturable organisms. Flow cytometry has become a powerful tool in microbiology, particularly for immunology, cancer research, food safety, and food preservation studies. Several commercial Live/Dead kits are available for applications in cell biology and microbiology (Hewitt et al., 1999; Barbesti et al., 2000). Antimicrobial peptide melittin activity was determined in three hours using flow cytometry to determine the number of *Streptococcus mutans*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Escherichia coli* that were killed by the peptide. The flow cytometric method was more efficient for determining the antibacterial activity of melittin on tested bacteria compared with

traditional methods of minimum inhibition and bactericidal concentration (MIC and MBC) (O'Brien-Simpson et al., 2016)

qPCR- Propidium Monoazide (qPCR-PMA)

PCR and Real- time quantitative PCR are widely used techniques with many applications in various laboratories. These molecular methods are considered rapid, highly sensitive, and accurate. They have been used for detection of pathogenic fungi, bacteria and viruses in several studies (López et al., 2003; McCartney et al., 2003). However, PCR and qPCR have several limitations, one of which is the occurrence of false positive results due to detection of naked DNA or RNA and nonviable microorganisms. The viability of microorganisms is a critical factor in food safety, quantification of organisms in infected samples and in risk assessment. Screening of compounds for antimicrobial activity mostly involves traditional bioassays including agar disk diffusion, well diffusion and broth agar dilution (Balouiri et al., 2016). Although these methods are able to distinguish between live and dead cells affected by tested compounds, they are time consuming due to long incubation periods and the need for appropriate selection of growth media for different groups of microorganisms. Obviously, these methods are not suitable for obligate parasites.

Viable cells possess intact membranes and dead cells most likely have damaged membranes. A DNA intercalating probe such as propidium monoazide (PMA) can only penetrate a compromised cell membrane (Nocker et al., 2006). The PMA dye contains azide that can be photo-activated and mask the DNA upon exposure to a light source, thus preventing the amplification of DNA by PCR. Recent studies have shown that PMA combined with qPCR is a promising method for differentiating live and dead cells of *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* (Kobayashi et al., 2009; Kim and Ko, 2012; Kacem et al., 2016).

OBJECTIVES

The overall objectives of this study are to obtain more understanding of the postharvest diseases of tomato and to evaluate potentially useful natural products and inorganic salts for disease management.

Specific objectives:

1. Determine the prevalent postharvest diseases of tomato on Oahu and identify the most virulent strains of pathogens associated with each disease.
2. Evaluate different natural products and inorganic salts for antifungal and antibacterial activity.
3. Evaluate antimicrobial activity of borate and its potential for reducing soft rot caused by *Pectobacterium carotovorum* on tomato fruit
4. Evaluate pre-harvest sprays of natural products and inorganic salts for reducing gray mold disease in the greenhouse.
5. Develop a specific a detection method for *Pectobacterium* using Recombinant Polymerase Amplification (RPA).

CHAPTER II

POSTHARVEST DISEASES OF TOMATO AND NATURAL PRODUCTS USED FOR DISEASE MANAGEMENT

Introduction

Public concern about fungicide residues on raw fruits and vegetables has stimulated research efforts using natural products to reduce the incidence of postharvest diseases. Approximately 25 and 38% of harvested fruits and vegetables, respectively, are lost to postharvest spoilage in the U.S. and global markets (Kantor et al., 1997). Fresh fruit and vegetables can be infected by pathogenic fungi and bacteria during crop growth in the field, harvesting, postharvest, storage and marketing (Barth et al., 2009). Postharvest diseases cause further economic losses, because of added costs of harvesting, transportation, and storage (Adikaram, 1986). The current study focuses on tomato (*Solanum lycopersicum* Mill), one of the most important vegetables produced globally, comprising approximately 14% of world vegetable production (Kader, 2004; USDA-ERS, 2016). The production value of tomato, estimated at more than \$50 billion, makes it the fourth most important commercial crop in the world (Vincent et al., 2013). Tomato is also one of the leading vegetable crops on Oahu, Hawaii. The first objective of this work was to determine the most serious postharvest diseases of tomato on Oahu and to identify the most virulent pathogens associated with each disease. The second objective was to evaluate natural products for their biocidal activity against pathogenic fungi and bacteria associated with tomato postharvest diseases.

Materials and Methods

Survey of postharvest diseases in tomato fruit

A survey was conducted on Oahu, Hawaii extending from November 2014 to April 2015. Samples of infected tomato were randomly collected from 17 locations and 37 markets on Oahu. Two samples (each sample consisting of 10 fruit) were selected from each market. Tissues showing symptoms of postharvest disease were cultured to identify associated pathogens. The percentage of infected tomato based on origin (local or imported) was reported.

Isolation and identification of pathogens

Small (1cm) sections of infected fruit were cut and surface-sterilized individually in 2% sodium hypochlorite for 1 min and rinsed twice in sterile distilled water. The pieces were dried between sterile Whatman No.1 filter paper and cultured on water agar plates and incubated at $28\pm 2^{\circ}\text{C}$ for 24 h. Single hyphal tips were transferred to Petri dishes containing V8- medium and incubated at $28\pm 2^{\circ}\text{C}$ for 5 days under a 12 h photoperiod (Carisse and Van Der Heyden, 2015). Purified cultures were visually identified (Dugan, 2006). For bacterial isolates, small sections of rotted tissues were suspended in distilled water for two minutes and the suspension was streaked onto the surface of nutrient agar (NA) plate and plates incubated at 30°C for 24 hours. Basic bacteriological tests including KOH sensitivity, oxidation/fermentation (OF), production of catalase, degradation of sodium polypectate, and hydrolysis of esculin and starch were conducted on each isolate. All bacterial strains were maintained -80°C until used. Presumptive identifications were confirmed with 16S rDNA sequence analysis (Weisburg et al., 1991).

Pathogenicity tests

Pathogenicity tests were performed on all fungal and bacterial isolates on three types of tomato fruit as previously described (Ahmed et al., 2016). Fruit were selected to be uniform in size and color, free from wounds and showing no symptoms of disease. Fruit were washed with tap water, surface "sterilized" by dipping in 1% sodium hypochlorite solution 10 min, rinsed by dipping twice in sterile distilled water for at least 10 min, and dried at ambient air temperature. A wound (1 mm diameter in 4 mm deep) was made on each fruit using a pipette tip. Mycelial plugs from 10-day-old-cultures of the fungal isolates were inserted into wounds using 0.2-10 µl pipette tips. For bacterial stains, fruit was inoculated with 20 µl of a bacterial suspension (1×10^8 /CFU). Inoculated fruit were placed in a plastic box containing sterile paper towels moistened with sterile water and incubated for 72 h at 23°C. The bacterium was recorded as pathogenic if symptoms of rot appeared on the tested fruit. The experiments were set up separately for fungal and bacterial isolates with four replications and each experiment was repeated twice.

Virulence tests

Tests were conducted to determine the most virulent isolates on each of three types of tomato fruit (common market, cherry, and grape). Fruit were selected to be uniform in size and color, free from wounds and showing no symptoms of disease. Virulence of each isolate was determined by measuring the lesion diameter of inoculated fruit after incubation at 23°C for 72 h. The experiments were set up as Complete Randomized Design (CRD) with four replicates. Data were analyzed using (SAS 9.2 V.USA) and means were compared by Duncan's multiple range tests. Differences at $p < 0.05$ were considered significant. The tests were repeated twice.

Molecular identification

Fungal DNA was extracted from freshly collected mycelium of 10-day-old cultures using the Microbial DNA Isolation Kit (MO BIO, Laboratories, Inc.). The ITS region of the fungal isolates was amplified with the primer pair ITS3 (5-GCATCGATG AAG AAC GCAGC-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3) (Nikolcheva et al., 2003; Ahmed et al., 2016).

Bacterial DNA was extracted from overnight cultures using the Microbial DNA Isolation Kit (MO BIO, Laboratories, Inc.) according to manufacturer's instructions. The 16S rRNA was amplified by PCR for all the isolates using the primers: 16S forward primer (5'-AGAGTTTGATCCTGGCTCAG-3) and 16S reverse primer (5'ACGGCTACCTTGTTACGACTT-3'). PCR was performed as previously described by (Weisburg et al., 1991; Srinivasa et al., 2012). Each PCR reaction was run with a negative control (no DNA). The PCR products were electrophoresed on 1.5 % agarose gels, stained with 0.4 µg/ml ethidium bromide, and bands visualized with a UV illuminator.

Sequence analysis

Sequence analysis was conducted as described in our previous work (Ahmed et al., 2016). PCR product was cleaned utilizing ExoSAP-IT (Affymetrix, Inc., USA). The 5 µl of post-PCR reaction and 2 µl ExoSAP-IT reagents were mixed. The mix was incubated at 37°C for 15 min following by incubation at 80°C for 15 min. Each purified template was sequenced on both strands using two flanking primers (ITS3- ITS4) for fungal isolates and 16s primers for bacteria. The sequences of ITS 3 and 4 regions, 16s of the tested isolates were edited in order to generate a consensus sequence from forward and reverse sequence in the amplicon using sequence assembly software (DNA BASER). A consensus sequence was analyzed by NCBI BLAST database for fungal and bacterial identities.

Natural controls

Leaf extracts made from *Capsicum annuum* cv. Stocky Red, *C. annuum* cv. Criolla de cocina, *C. chinense* cv. NuMexsuave, *Tagetes tenuifolia*, *Aloe vera* (leaves and gel), and *Origanum vulgare*, *Azadirachta indica* (Neem oil) and a proprietary formulation (APF) (Agrichem, Inc., Australia, <http://www.agrichem.com.au/>) were tested. Leaves were extracted by the method described by (Wilson et al., 1997) with the following modification. Raw leaves were collected in plastic bags and were frozen for a minimum of 12 h at 20°C. The plastic bag was removed and leaf fluids were sterilized by passing the liquid through a 0.22 µm Millipore filter and stored in 4°C until used.

Assays of antifungal activity

The antifungal activity of nine plant extracts was evaluated against *Alternaria* and *Botrytis* isolates by using an inhibition assay described as the inhibition assay (McCutcheon et al., 1994) with slight modification. A 5 ml sterilized crude extract was mixed with 15 ml of 45°C cooled molten V8 medium and allow solidifying at room temperature for 30 min. A mycelial disc 6 mm diameter of 7 to 10- day- old cultures was transferred to a Petri plate containing V8 and crude extract. The V8 plate without plant extract served as a control. The antifungal activity of APF was evaluated against *B. cinerea* (B03) at five concentrations 1, 0.5, 0.25, 0.125, 0.0652 ml/L. The most effective APF concentration was reevaluated for the remaining 33 pathogenic fungal isolates. Since different genera have different growth rates on V8 medium, a separate control was established for each fungus by recording the time needed for mycelium to reach the edge of the Petri dish. At that point, the corresponding plate containing APF was measured for inhibition. The experiments were conducted as Complete Randomized Design (CRD) with four replications. Data were analyzed using (SAS 9.2 V.USA). Means were compared by Duncan's

multiple range tests. Differences at $p < 0.05$ were considered significant. Each experiment was repeated three times.

Transmission Electron Microscopy

The possible damage to fungal membranes treated with and without APF was assessed using TEM following the method of Al-Adham et al (2000) with slight modification. Spores of *B.cinerea* were collected from three-days-old culture grown on V8 media plate. Spores were grown on potato dextrose broth supplemented with or without APF 1ml/L for 2 and 6h at 28°C. One-ml aliquots were centrifuged at 4000 x g for 10 min at room temperature and washed two times with sterile distilled water. The pelleted cells were fixed with 4% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2, held at room temperature overnight and washed in 0.1M cacodylate buffer 2 x for 10 min, followed by postfixation with 1% OsO₄ in 0.1M cacodylate buffer for 1h. Cells were dehydrated in a graded ethanol series (30, 50, 70, 85, 95, and 100%), substituted with propylene oxide, and embedded in LX112 epoxy resin and placed in a 60°C oven for 24h to polymerize the resin. Ultrathin (60-80 nm) sections made on a resin-fixed pellet using RMC PowerTome Ultramicrotome and double stained with uranyl acetate and lead citrate. Sections were examined utilizing a Hitachi HT7700 TEM (Hitachi, Tokyo, Japan) at 100 kV and photographed with an AMT XR-41B 2k x and 2k CCD camera.

Results

Survey

Ninety-nine fungal and seventeen bacterial isolates were recovered from tomato from the 37 markets on Oahu (Figure 2.1). Fungal genera were *Alternaria*, *Botrytis*, *Colletotrichum*, *Fusarium*, *Geotrichum*, *Mucor*, *Stemphylium*, *Rhizopus*, and *Penicillium*. Bacterial genera were *Acetobacter*, *Gluconobacter*, *Klebsiella*, *Leuconostoc*, and *Pectobacterium* (Figure 2.2 & Table

2.1). Examination of diseased tomatoes based on origin showed 51% were imported from California and Mexico and 49% had been grown locally at three sites on Oahu.



Figure 2.1. Map of Oahu showing sites where fruit samples were collected. Numbers refer to geographic divisions on the island of Oahu. Dots show sites where fruits were collected from separate markets. <http://katebraden.com/neighborhoods.shtml>.

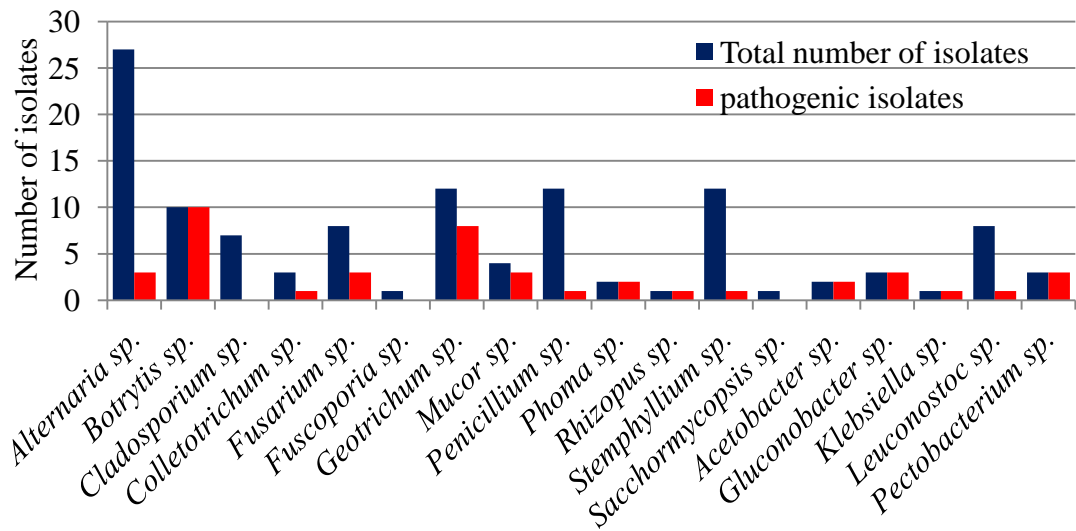


Figure 2.2. Genera of fungi and bacteria isolated from infected tomato fruit during a market survey on Oahu.

Table 2.1. Characteristics of bacterial isolates obtained from infected tomato fruit.

Bacterial strains	Strain No.	Gram ^a	O/F	Pectolytic	Esculin	Starch	Catalase
<i>Gluconobacter frateurii</i>	BA01	-	+/+	-	-	-	+
<i>Klebsiella oxytoca</i>	BA02	-	+/+	-	+	-	+
<i>Leuconostoc</i> sp.	BA03	+	+/-	-	+	-	+
<i>Leuconostoc</i> sp.	BA04	+	+/-	-	+	-	+
<i>Acetobacter</i> sp.	BA05	-	+/+	-	-	-	-
<i>Leuconostoc</i> sp.	BA06	+	+/+	-	-	-	+
<i>Leuconostoc</i> sp.	BA07	+	+/+	-	-	-	+
<i>Leuconostoc</i> sp.	BA08	+	+/-	-	-	-	-
<i>Gluconobacter frateurii</i>	BA09	-	+/+	-	-	-	+
<i>Leuconostoc</i> sp.	BA10	+	+/+	-	+	-	+
<i>Leuconostoc</i> sp.	BA11	+	+/+	-	+	-	+
<i>Pectobacterium carotovorum</i>	BA12	-	+/+	+	+	-	+
<i>Acetobacter</i> sp.	BA13	-	+/+	-	-	-	+
<i>Gluconobacter frateurii</i>	BA14	-	+/+	-	-	-	+
<i>P.carotovorum</i>	BA15	-	+/+	+	+	-	+
<i>Leuconostoc citreum</i>	BA16	+	+/+	-	-	-	-
<i>P.carotovorum</i>	BA17	-	+/+	+	+	-	+

a) Presumptive Gram stain determined by the formation of sticky stands 30s after immersing in 3% KOH which is an indication of a Gram negative for bacterial cells.

b) Pectolytic enzyme production

c) Esculin hydrolysis

d) Starch hydrolysis

e) Catalase production

Pathogenicity and virulence

The pathogenicity tests showed that 33 of 99 fungal isolates and 10 of 17 bacterial isolates were pathogenic on all three types of tomato fruit (Table 2.2). Other reports indicate that fungi and bacteria can survive and grow saprophytically on tomato (Smilanick, 2004; Agrios, 2005a). In this study, the fungal isolates varied in virulence. The pathogenic isolates of *B. cinerea* showed greater lesion diameter range with 16-70, 10-32, and 10-25 mm on three types of tomato fruit, common market, cherry and grape, respectively (Table 2.3). In addition, *B. cinerea* isolates varied in their standard deviations, indicating that *Botrytis* isolates had different level of virulence. *B. cinerea* (B03) and *P. carotovorum* (BA17) were significantly more virulent than other isolates when tested on the common market, cherry and grape tomato (Tables 2.4 & 2.5). All *Botrytis* and *Pectobacterium* isolates were pathogenic on the original host from which they were isolated (Figure. 2.2). *B. cinerea* (B03) and *P. carotovorum* (BA17) produced significantly larger lesion diameters (Tables 2.4 & 2.5).

Molecular Identification

A PCR product with expected size 370 bp was amplified successfully for all fungal isolates (Figure. 2.3). A NCBI BLAST web (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for the ITS3-ITS4 region to determine the fungal identity and found that sequence maximum identity of > 98% with other *B. cinerea* entries. In addition, the BLAST results of *Botrytis* isolates B03 and B09 matched $\geq 99\%$ homology to *B. cinerea* (370 base pairs).

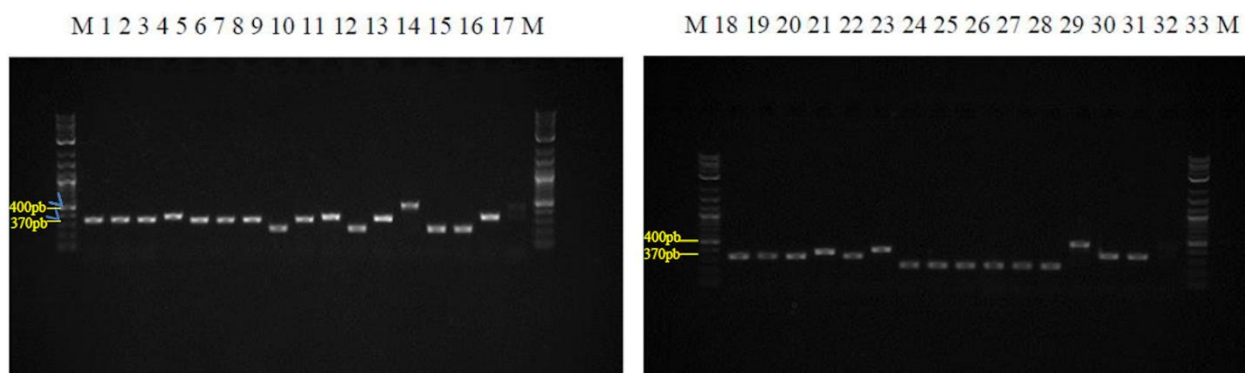


Figure 2.3. Amplified PCR products following gel electrophoresis using DNA from isolated fungi. M = Marker (0.1-10.0 kb); 1-3 = *Botrytis* sp.; 4 = *Rhizopus* sp.; 5-6 = *Botrytis cinerea*; 7 = *Botrytis* sp.; 8 = *Mucor* sp.; 9-10 = *Fusarium* sp.; 11 = *Penicillium*. 12 = *Stemphylium* sp.; 13 = *Colletotrichium* sp.; 14-15: = *Geotrichium* sp.; 16 = *Phoma* sp.; 17 = NC (No DNA); 18-20 = *Botrytis* sp.; 21 = *Alternaria* sp.; 22 = *Botrytis* sp.; 23 = *Fusarium* sp.; 24-29 = *Geotrichium* sp. 30 = *Phoma* sp.; 31-32 = *Mucor* sp., 33 = NC (No DNA).

Table 2.2. Pathogenicity tests of fungal and bacterial isolates on the artificially wounded fruit of three types of tomato 72h after incubation at 23°C.

Isolates	Isolate No.	Tomato variety		
		Common market	Cherry	Grape
<i>Alternaria</i> sp.	A01- A03, A05- A19, A21	-	-	-
<i>Alternaria</i> sp.	A04, A20, A22	+	+	+
<i>Botrytis cinerea</i>	B01- B10	+	+	+
<i>Colletotrichum</i> sp.	C01	+	+	+
<i>Colletotrichum</i> sp.	C02	-	-	-
<i>Fusarium</i> sp.	F01- F02, F04, F06	-	-	-
<i>Fusarium</i> sp.	F03, F05, F07- F08	+	+	+
<i>Geotrichum</i> sp.	G01- G03, G05- G11	+	+	+
<i>Geotrichum</i> sp.	G04, G06	-	-	-
<i>Mucor</i> sp.	M01- M02	-	-	-
<i>Mucor</i> sp.	M03, M04	+	+	+
<i>Penicillium</i> sp.	P07	+	+	+
<i>Penicillium</i> sp.	P01-P06	-	-	-
<i>Rhizopus</i> sp.	R01- R03	+	+	+
<i>Stemphylium</i> sp.	S01- S02	+	+	+
<i>Acetobacter</i> sp.	BA5, BA13	+	+	+
<i>Gluconobacter</i> sp.	BA1, BA9, BA14	+	+	+
<i>Klebsiella</i> sp.	BA2	+	+	+
<i>Leuconostoc</i> sp.	BA3- BA4, BA6- BA8, BA10- BA11	-	-	-
<i>Leuconostoc</i> sp.	BA16	+	+	+
<i>Pectobacterium</i> sp.	BA12, BA15, BA17	+	+	+

+ = rotting; - = no rotting

Table 2.3. The average, range and standard deviation of virulence fungal isolates on the common market, cherry and grape tomato 72h after incubation at 23°C.

Genus	Isolate No.	Tomato variety								
		Common market			Cherry			Grape		
		\bar{x}	R	SD	\bar{x}	R	SD	\bar{x}	R	SD
<i>Alternaria</i> sp.	3	29	28-30	1.15	17	13-20	3.49	12	12-13	0.50
<i>Botrytis cinerea</i>	10	48	16-70	19.53	20	10-32	7.03	16	10-25	5.43
<i>Colletotrichum</i> sp.	1	33	-	-	19	-	-	15	-	-
<i>Fusarium</i> sp.	3	35	32-38	2.84	21	20-21	0.76	18	18	0.41
<i>Geotrichum</i> sp.	8	39	35-40	1.87	20	18-25	2.11	20	20	3.29
<i>Mucor</i> sp.	3	56	48-60	6.92	21	20-22	1.07	20	20	0.44
<i>Penicillium</i> sp.	1	35	-	-	21	-	-	12	-	-
<i>Phoma</i> sp.	2	35	31-40	6.12	17	17-17	0.00	11	11-11	0.00
<i>Rhizopus</i> sp.	1	60	-	-	27	-	-	20	-	-
<i>Stemphylium</i> sp.	1	30	-	-	19	-	-	13	-	-

Table 2.4. Virulence of *Botrytis cinerea* isolates on the common market, cherry and grape tomato 72 h after incubation at 23°C.

Isolate No.	Tomato variety/Lesion diameter (mm)		
	Common market	Cherry	Grape
B01	68.00 ^a	27.00 ^b	20.00 ^b
B02	30.00 ^c	22.00 ^c	10.00 ^c
B03	70.00 ^a	32.00 ^a	25.00 ^a
B04	30.00 ^c	12.00 ^{de}	12.00 ^c
B05	30.00 ^c	16.00 ^d	10.00 ^c
B06	16.00 ^d	14.00 ^d	13.00 ^c
B07	59.00 ^b	22.00 ^c	18.00 ^b
B08	60.00 ^b	22.00 ^c	20.00 ^b
B09	61.00 ^b	25.00 ^{b^c}	20.00 ^b
B10	59.00 ^b	10.00 ^e	10.00 ^c
Distilled water	01.00 ^e	01.00 ^f	01.00 ^d

* Mean values followed by different letters within a column are different according to Duncan's Multiple Range Test ($p \leq 0.05$).

Table 2.5. Virulence of bacterial isolates on the common market, cherry and grape tomato 72 h after incubation at 23°C.

Bacterial strains	Strain No.	Tomato variety/Lesion diameter (mm)		
		Common market	Cherry	Grape
<i>Acetobacter</i> sp.	BA05	27.00 ^{de}	20.00 ^d	15.00 ^c
<i>Acetobacter</i> sp.	BA13	35.00 ^c	23.00 ^c	17.00 ^b
<i>Gluconobacter</i> sp.	BA01	30.00 ^d	21.00 ^d	15.00 ^c
<i>Gluconobacter</i> sp.	BA09	24.00 ^e	14.00 ^e	10.00 ^d
<i>Gluconobacter</i> sp.	BA14	30.00 ^d	14.00 ^e	11.00 ^d
<i>Klebsiella</i> sp.	BA02	26.00 ^e	12.00 ^f	11.00 ^d
<i>Leuconostoc</i> sp.	BA16	37.00 ^c	23.00 ^c	17.00 ^b
<i>Pectobacterium</i> sp.	BA12	47.00 ^b	29.00 ^b	24.00 ^a
<i>Pectobacterium</i> sp.	BA15	35.00 ^c	23.00 ^c	17.00 ^b
<i>Pectobacterium</i> sp.	BA17	66.00 ^a	31.00 ^a	24.00 ^a
Distilled water	control	01.00 ^f	01.00 ^g	01.00 ^e

* Mean values followed by different letters within a column are significantly different according to Duncan's Multiple Range Test ($p \leq 0.05$).

Inhibitory Effect of Plant Extracts on Fungal colony

The crude leaf extracts of *C. annuum* cv. ‘StockyRed’, *C. annuum* cv. ‘Criolla de cocina’, *C. chinense* cv. ‘NuMexsuave’, *T. tenuifolia*, *A. vera*, *O. vulgare* and *A. indica* Neem oil showed no measurable inhibition of mycelial growth for *Alternaria* sp. or *Botrytis* sp. In contrast, APF completely inhibited mycelial growth of both fungi (Figure 2.4). The most effective APF concentration was (1 mL/l) that completely inhibited the growth of all 33 of the other tested pathogenic fungi (Table 2.6).

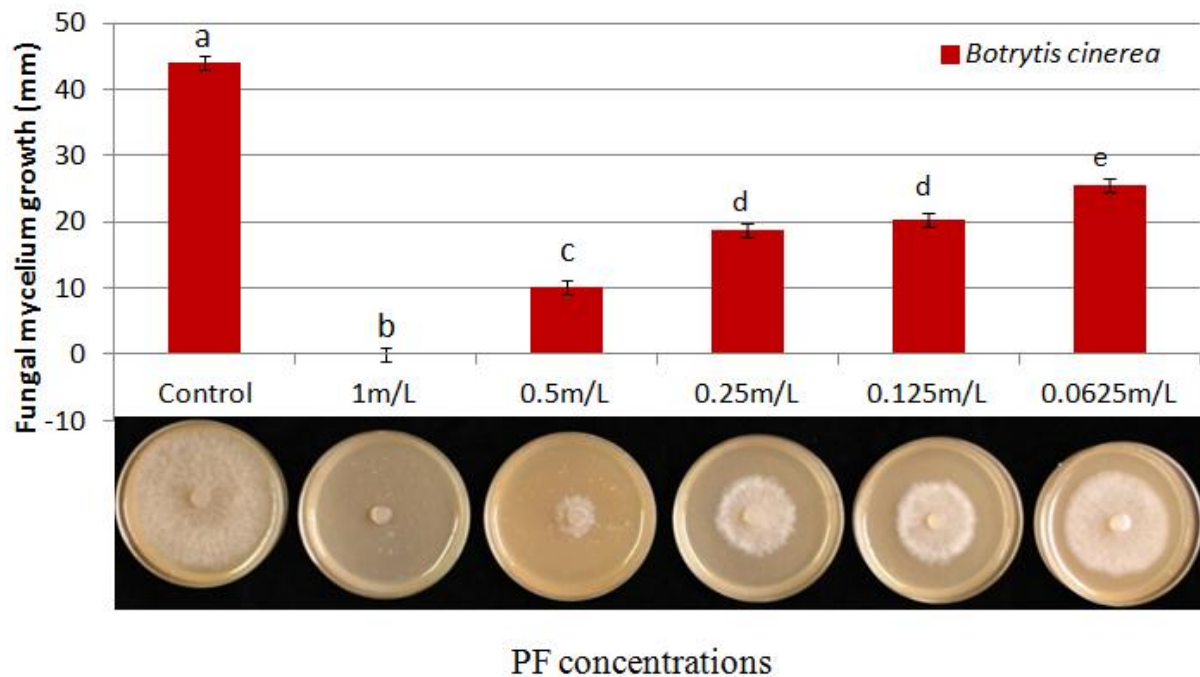


Figure 2.4. The mycelial growth of *Botrytis cinerea* (B03) on agar containing a proprietary product (APF) added to the culture plates at five different concentrations.

Table 2.6. Effect of APF (1 ml/L) on mycelial growth of 33 pathogenic fungi 72h after incubation at 23°C using an inhibition assay.

Fungal isolates	Isolate No.	Colony diameter (mm)*
Control (V8 only)	---	44.00 ^a
<i>Alternaria</i> sp.	A04	00.00 ^e
<i>Alternaria</i> sp.	A20	00.00 ^e
<i>Alternaria</i> sp.	A22	00.00 ^e
<i>Botrytis</i> sp.	B01	03.00 ^c
<i>Botrytis</i> sp.	B02	00.00 ^e
<i>Botrytis</i> sp.	B03	00.00 ^e
<i>Botrytis</i> sp.	B04	00.00 ^e
<i>Botrytis</i> sp.	B05	04.00 ^b
<i>Botrytis</i> sp.	B06	00.00 ^e
<i>Botrytis</i> sp.	B07	04.00 ^b
<i>Botrytis</i> sp.	B08	00.00 ^e
<i>Botrytis</i> sp.	B09	00.00 ^e
<i>Botrytis</i> sp.	B10	00.00 ^e
<i>Colletotrichum</i> sp.	Col1	00.00 ^e
<i>Fusarium</i> sp.	F03	00.00 ^e
<i>Fusarium</i> sp.	F07	02.00 ^d
<i>Fusarium</i> sp.	F08	00.00 ^e
<i>Geotrichum</i> sp.	G01	00.00 ^e
<i>Geotrichum</i> sp.	G02	00.00 ^e
<i>Geotrichum</i> sp.	G03	00.00 ^e
<i>Geotrichum</i> sp.	G05	00.00 ^e
<i>Geotrichum</i> sp.	G06	00.00 ^e
<i>Geotrichum</i> sp.	G07	00.00 ^e
<i>Geotrichum</i> sp.	G08	00.00 ^e
<i>Geotrichum</i> sp.	G09	00.00 ^e
<i>Mucor</i> sp.	M01	00.00 ^e

Table 2.6. Continued

Fungal isolates	Isolate No.	Colony diameter (mm)*
<i>Mucor</i> sp.	M03	00.00 ^e
<i>Mucor</i> sp.	M04	00.00 ^e
<i>Penicillium</i> sp.	P07	00.00 ^e
<i>Phoma</i> sp.	Ph01	00.00 ^e
<i>Phoma</i> sp.	Ph02	00.00 ^e
<i>Rhizopus</i> sp.	R01	03.00 ^c
<i>Stemphyllium</i> sp.	S01	00.00 ^e

* Mean values followed by different letters within a column are significantly different according to Duncan's multiple range test ($p \leq 0.05$).

Effect of APF on fungal spores Analyzed by TEM

Transmission electronic microscopy was used to visualize morphological changes in *B.cinerea* spores treated with 1ml/L APF for 2 and 6 h at $25\pm 2^{\circ}\text{C}$. Intact cell wall, cell membrane of spores, and cytoplasm were normal showing no obvious changes in morphological structure in the untreated control sample of *Botrytis cinerea* spores (Figure 2.5 A). In contrast, fungi grown in nutrient broth supplemented with 1ml/L APF showed distinct signs of deterioration, deformation or condensed cytoplasm (Figure 2.5 B) and the condensation of cytoplasm was greater upon longer exposure of fungal spores to APF for 6h (Figure 2.5 C).

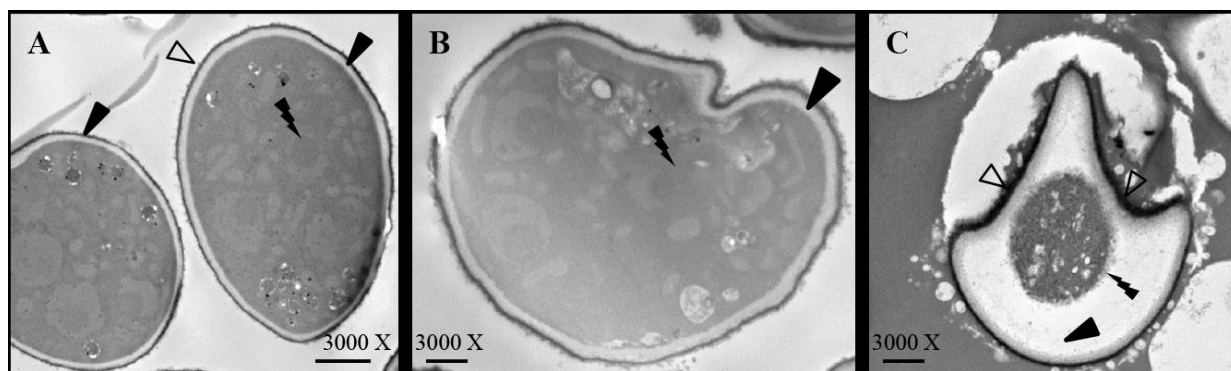


Figure 2.5. Micrographs of *Botrytis cinerea* spores examined with transmission electron microscopy. Spores were incubated in potato dextrose broth (PD) without Agrichem propriety formula (APF) (A) or with 1ml/L APF for 2 h (B) and 6 h (C). In the absence of APF, the cytoplasm (black lightening bolts), cell wall (clear arrows) and cell membranes (black arrows) were intact and appeared normal. Following treatment with 1ml/L APF the cell membranes were degraded, the cytoplasm became condensed and cell structure was abnormal.

Discussion

Some of pathogens obtained during the survey are known to survive on fruit and be spread during transportation, handling, and storage (Barkai-Golan, 2001a). Differences in virulence among pathogens are frequently a result of the differences in production of cell wall degrading enzymes (CWDEs), oxalic acid and/or secretion of pathogenicity factors (Bellincampi et al., 2014; Kubicek et al., 2014a). In previous studies, *Botrytis* and *Pectobacterium* were some of the most important pathogens causing spoilage decay on tomato (Etebu et al., 2013; Akbar et al., 2014; Fillinger and Elad, 2015; Ahmed et al., 2016). Transmission electron microscopy showed degradation of fungal spore- wall; condensed cytoplasm, and abnormal spore structure, indicating that treated spores were damaged by APF.

Conclusion

Thirty percent of the fungal and 58% of the bacterial isolates were pathogenic. *B. cinerea* and *P. carotovorum* were the most virulent postharvest pathogens of tomato on Oahu.. A natural proprietary product (APF) had sufficient antifungal activity to completely inhibit mycelial growth of all isolated fungi but had no effect on the bacteria. This natural product is a potential alternative to synthetic fungicides in reducing postharvest gray mold disease.

CHAPTER III

NATURAL PRODUCTS USED TO CONTROL POSTHARVEST GRAY MOLD OF TOMATO FRUIT- POSSIBLE MECHANISMS

Introduction

Tomato (*Solanum lycopersicum* Mill) is one of the most important vegetables produced globally. Fresh tomato production is the highest of all vegetable crops in the U.S and the U.S. is the main world producer of tomato (USDA-ERS, 2016). However, postharvest diseases of fruit and vegetables cause major losses in food production. Approximately 23-25% and 31-38% of harvested fruit are lost to postharvest spoilage in the USA and the world respectively (Kantor et al., 1997b). Gray mold, caused by *Botrytis cinerea*, is considered one of the most destructive postharvest diseases of tomato in both field and greenhouse production where the temperature and humidity are conducive to fungal development (Elad et al., 2007). *B. cinerea* infection of tomatoes causes major economic losses at the pre- and post-harvest stages (Soylu et al., 2010). Currently, synthetic fungicides are commonly used to control postharvest infections of *B. cinerea* (Mari et al., 2014).

Increasing international concerns for public health, excessive use of pesticides and development of fungal strains resistant to fungicides have resulted in the introduction of regulations and policies to limit the use of synthetic pesticides over the past decades (Holmes and Eckert, 1999; Liu et al., 2013). Alternative, economically feasible approaches are needed to reduce postharvest gray mold of tomato fruit. Toward this end, antimicrobial characteristics of various plant extracts have been described for use in plant protection (Campo et al., 2000). Many applications of substances, such as chitosan, extracts of *Azadirachta indica* seed, essential oils, medicinal plants, and mineral nutrients such as selenium and borate have been investigated for

the control of postharvest fruit spoilage and several have been successfully applied (Bautista-Ban et al., 2006; Emma et al., 2013; Ibrahim and Al-Ebady, 2014b; Wu et al., 2016).

Plants in the family Solanaceae have antimicrobial and antifungal properties (Bosland, 1996; Dorantes et al., 2008; Omolo et al., 2014). Pepper plants (*Capsicum* sp.) also have a wide range of uses, including pharmaceutical, natural pigment agents, cosmetics, and as ornamentals Brito-Argáez et al., 2009). Phytochemical analysis of *Capsicum* species demonstrated that the presence of tannins, alkaloids, steroids, glycosides, flavonoids, phenols and terpenoid, provided a wide range of antimicrobial activities (Vinayaka et al., 2010; Gayathri et al., 2016).

Waltheria indica is a woody plant belonging to the family Malvaceae found widely in tropical and subtropical regions including Hawaii (Abbott and Shimazu, 1985), South America (Olajuyigbe et al., 2011), and South Africa (Mathabe et al., 2006). Its antibacterial and antifungal activity has been documented in several studies (Almagboul et al., 1988; Garba et al., 2012). The phytochemical investigation from the crude extract of *W. indica* indicated the presence of several active chemical compounds such as alkaloids, flavonoids, tannins, sterols, terpenes, and saponins (Zongo et al., 2013). Scant information is available regarding the antimicrobial activity of pepper and *Waltheria* extracts on postharvest fungi or their modes of action.

In the present study, leaf extracts of *Capsicum chinense* (Datil), *C. annuum* (Carnival), *C. frutescens* (Hawaiian chili pepper), and *Waltheria indica* (sleepy morning) were tested as potential natural antifungal agents using spore germination of *B.cinerea* as a measure. The role of intercellular reactive oxygen species (ROS) and the integrity of the plasma membranes in relationship to germination of *B. cinerea* spores were also examined.

Materials and Methods

Fungal isolates

Fungi were isolated from diseased tomato fruit collected from 37 markets located throughout the island of Oahu, Hawaii. Small sections (1 mm²) were excised from a range of lesions, incubated on water agar. Single hyphal tips were transferred to V-8 agar in 9 cm Petri dishes and incubated for 10 to 14 d at 20°C with a 12-h photoperiod (Carisse. and Heyden., 2015). The isolates were stored at 2°C in the sterile soil for further study. Fresh cultures were established as needed by placing 0.2 g soil on V-8 Petri plates. Ten isolates of *B. cinerea* were selected for further study.

Pathogenicity tests

Tests were conducted to determine the most virulent isolates of *B. cinerea* on tomato fruit (common market, cherry and grape tomatoes), detached leaves and whole plants. Fruit were inoculated with mycelium of 10-day-old-cultures of the selected isolates of *B. cinerea* using 0.2-10µl pipette tips. On the detached leaves, 6-mm fungal plugs were placed on 4-week-old surface sterilized tomato leaves placed in 9-cm-d Petri dishes including wet filter paper. The Petri plates were held for 72 h at 23°C in the dark. On tomato plants, spore suspensions of 1x10⁵ spores/ml from 10-day-old cultures of each isolate of *B. cinerea* were prepared using a hemocytometer. Fungal spore suspensions were sprayed on 4-week-old tomato plants of cv. Favorite, F1 (Johnny selected seeds, Winslow, ME) growing in 10-cm-d plastic pots. The plants were placed in plastic bags, sealed, and held for 24 h at 23°C. Plastic bags were removed and plants returned to the greenhouse for 14 days. For disease assessment of tomato fruit and detached leaves, the lesion diameter of inoculated fruit and leaves were measured after 72 h at 23°C. On tomato plants, the disease severity was scored on diseased plant utilizing a 0-5 scale.

The experiments were set up as Complete Randomized Design (CRD) with four replications. Data were analyzed using (SAS 9.2 V.USA) and means were compared by Duncan's multiple range tests. Differences at $p < 0.05$ were considered significant. All experiments were repeated three times.

Plant extraction and multi-well plates

Leaf extracts were made from *C. chinense* cv. Datil, *C. annuum* cv. Carnival, and *C. frutescens* cv. Hawaiian chili pepper obtained from Waimanalo Research Station and *W. indica* obtained from H1/University Ave (Native Hawaiian Plant Demonstration Site). Plant materials were extracted by the following method described by Wilson et al., (1997) with some modification. Fresh plant material (100 g) was collected in resealable plastic bags (20 x 20 cm) and placed in a freezer for a minimum of 12 h at -20°C . As plant material was tested, it was withdrawn from the freezer and allowed to thaw for a minimum of 20 min. Freezing and thawing fractured the plant cells. The plastic bag was tilted so that the fluid collected in one corner, and extracted plant fluids collected in plastic weight boats. The extract was filter-sterilized by passing through a $0.22\ \mu\text{m}$ Millipore filter. Filtered 10, 20, 30, 40% extract solutions were prepared by adding 1, 2, 3, and 4 ml of sterile extract with 9, 8, 7, and 6 ml respectively of a *B. cinerea* spore suspension (1×10^5 conidia/ml) in sterile malt extract broth.

Fifty μl of the plant extract and spore suspension mixture was pipetted into each well of a row of a 96 multi-well plate. A nontreated spore suspension was added to one row of each plate for comparison, and a blank row was included as an instrument check. Each well was sealed with a sheet of parafilm to prevent cross-contamination by volatiles. After 6, 12, 24 h incubation at 25°C , the density of fungal growth in the wells was measured with an Epoch microplate reader (BioTek Instrument, Inc.). Absorbency was measured for each well using a 492-nm filter.

Background readings from the average of the nontreated samples were subtracted by the Gene5 software program to provide net fungal growth in tested samples.

Reactive oxygen species (ROS)

A method using fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) was used with slight modifications to assess the intercellular level of ROS in *B. cinerea* (Wu et al., 2016). Spores of *B. cinerea* were cultured in malt extract broth supplemented with 40% *C. chinense* or *C. annuum* leaf extract and incubated for 2, 4, and 6 h at 23°C. The spores of 1×10^5 per ml were collected and washed with 10 mM potassium phosphate buffer (pH 7.0) and incubated for 1 h in the same buffer containing 10 μ M DCFH-DA (dissolved in dimethyl sulfoxide). After washing twice with potassium phosphate buffer, spores were examined under a Zeiss microscope (Axioscop.A1, USA) using a fluorescein 2, 7- dichlorodihydro-specific filter. At least 100 spores were examined for each treatment with three replicates.

Membrane integrity

A propidium iodide (PI) staining method was used to detect the membrane integrity of *B. cinerea* conidia (Wu et al., 2014). Spores of *B. cinerea* were cultured in malt extract broth medium supplemented with 40% of *C. chinense*, *C.annuum* leaf extract and incubated 2, 4, and 6 h at 23°C. The spores of 1×10^5 per ml were collected and stained with 10 μ g/ml PI for 5 min at 30°C. The spores were centrifuged and washed twice with 10 mM potassium phosphate buffer (pH 7.0) to remove residual dye. The spores were observed under a Zeiss microscope (Axioscop.A1, USA) and red stained conidia were recorded. At least 100 spores were examined for each treatment with three replicates.

Transmission electron microscopy (TEM)

The possible damage to fungal cell wall and membranes treated with and without *Capsicum chinense* or *C. annuum* extracts was assessed using TEM following the method of Al-Adham et al., (2000) with slight modification. Spores of *B. cinerea* were collected from three-day-old culture grown on V8 media plate. Spores were grown in potato dextrose broth supplemented with or without each plant extract (40 %) for 2 or 6h at 28°C. One-ml aliquots were centrifuged at 4000 x g for 10 min at room temperature and washed two times with sterile distilled water. The pelleted cells were fixed with 4% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2, held at room temperature overnight and washed twice with 0.1M cacodylate buffer 2 x for 10 min, followed by postfixation with 1% OsO₄ in 0.1M cacodylate buffer for 1h. Cells were dehydrated in a graded ethanol series (30, 50, 70, 85, 95, and 100%), substituted with propylene oxide, and embedded in LX112 epoxy resin and placed in a 60°C oven for 24h to polymerize the resin. Ultrathin (60-80 nm) sections made on a resin-fixed pellet using RMC PowerTome Ultramicrotome and double stained with uranyl acetate and lead citrate. Sections were examined utilizing a Hitachi HT7700 TEM (Hitachi, Tokyo, Japan) at 100 kV and photographed with an AMT XR-41B 2k x and 2k CCD camera.

Effects of plant extracts on gray mold disease of tomato fruit

Protective action of plant extracts

Tomato fruit that were uniform in size and color, free from wounds and rot were selected. The fruits were washed with tap water; surface sterilized by dipping in 1% sodium hypochlorite solution for 10 min, rinsed by immersing in two changes of sterile distilled water, and dried in ambient air. A wound 1 mm diameter and 4 mm deep was made on each fruit using a pipette tip. Wounded fruit were immersed for 10 min in the different *Capsicum chinense* and *C. annuum*

extracts at (0-, 40-, and 60 %) concentrations containing natural sticking agent TX 70 (600 µl/L) (OMRI, OR, USA) for 1-, 2-, 5- and 10- min, and then dried in ambient air. Each wound was inoculated with a 10-µl aliquot containing 1×10^5 spores/ml of *B. cinerea* (Chen et al., 2011). Fruit were placed onto wet paper towels in a plastic container and incubated for 3 days at 24°C. Disease severity was assessed by measuring lesion diameter on each fruit. Each treatment contains five fruit. Lesion diameter was the mean of the five fruit in each replicate. The experiment was repeated twice.

Curative action of plant extracts

A curative assay was conducted using the procedures used for the protective assay except that the wounded fruit were immersed in the plant extracts for 12 h after inoculation (Yaganza et al., 2014). Each treatment was replicated by five fruits and the test was repeated twice.

Statistical analysis

All statistical analyses were performed using SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). Data were subjected to one- way analysis of variance (ANOVA). Where appropriate, means were compared using Duncan's Multiple Range Test. Differences at $P < 0.05$ were considered significant.

Results

Ten *Botrytis* isolates were made from 33 pathogenic-fungal genera recovered from infected tomato in a previous survey. Based on the morphological characteristics of isolates, microscopic observations of their conidiophores and conidia, all were identified as *Botrytis* spp.

Pathogenicity tests

The ten isolates of *Botrytis* spp. were highly variable with respect to the disease on tomato fruit, detached leaves, and whole plant. The lesion diameter ranged from 70, 32, and 25 to 16, 10, 10 mm on common market, cherry, and grape tomato, respectively; lesion size ranged from 28 to 14 mm on detached leaves; and the severity scale ranged from 1 to 5 on whole plants, showing wide pathogenic variability. On tomato fruit, gray mold development differed on the common market, cherry and grape tomatoes. All isolates were pathogenic on the original host from which they were isolated. The largest lesion diameters on inoculated fruit were caused by *B. cinerea* B03 ($p < 0.05$) in comparison to other isolates and the control (Figure 3.1). Necrotic lesions developed on detached leaves after 3 d for all isolates. Lesion diameters differed among *Botrytis* isolates with lesion size of B03 and B09 being greater ($p < 0.05$) when compared with other isolates (Figure 3.2). On whole tomato plants, isolate B03 was the most virulent among all the isolates 14 days after inoculation (Fig. 3.3). B08 and B09 were less virulent on tomato plants. Other isolates varied in their disease severity. Since *B. cinerea* isolate B03 was the most virulent isolate; it was used for further study.

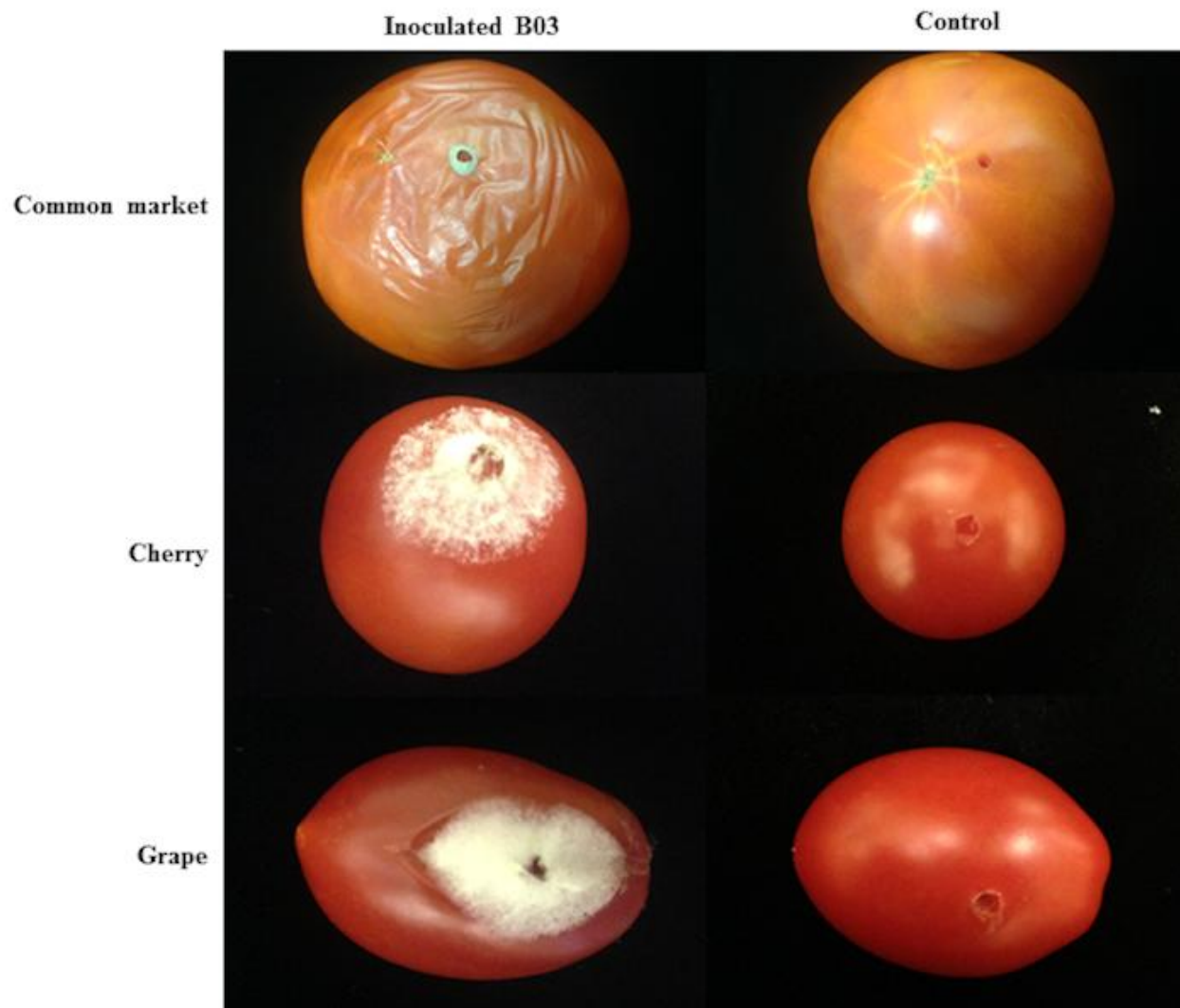


Figure 3.1. Lesions on tomato fruit inoculated with *Botrytis cinerea* B03

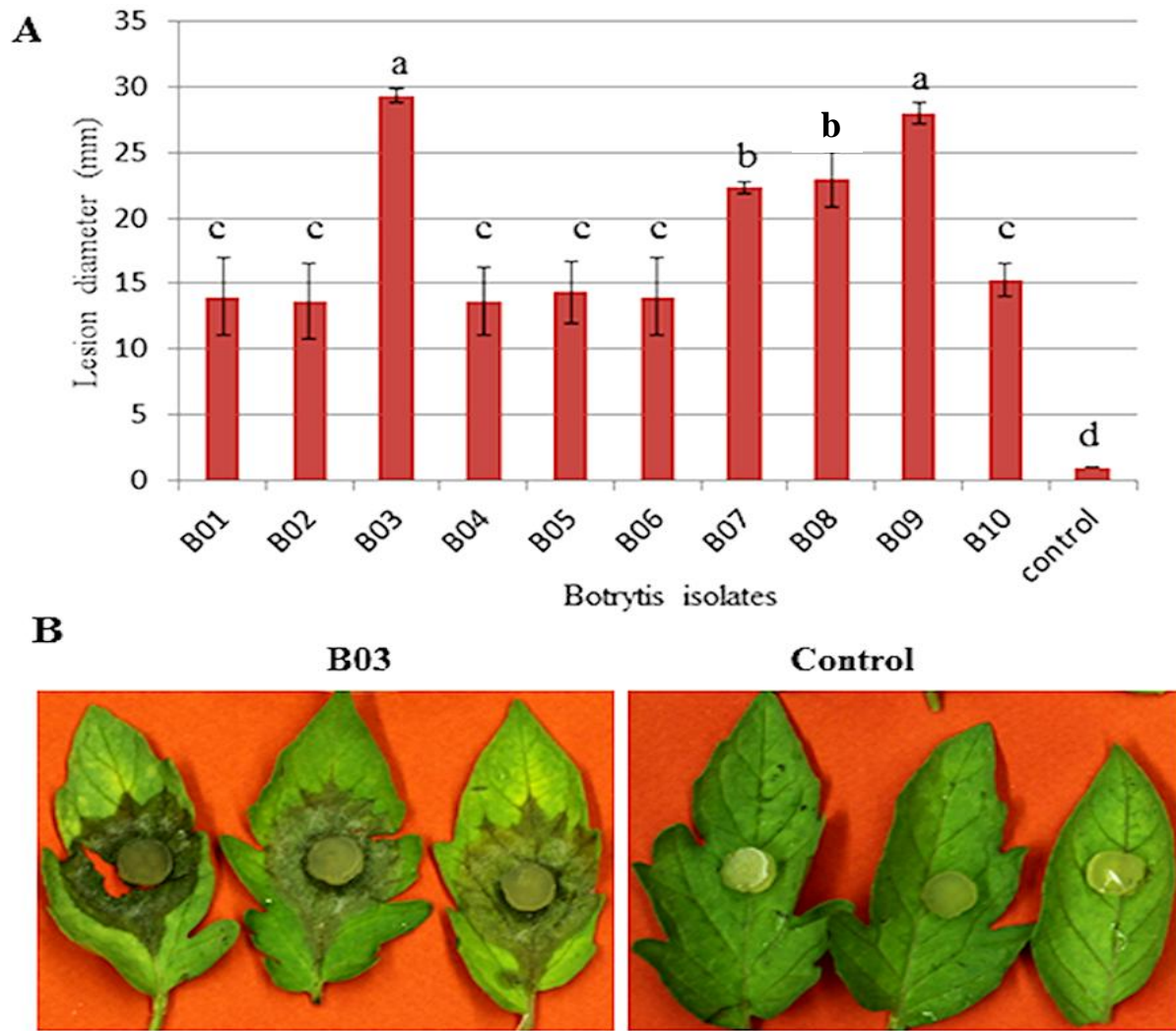


Figure 3.2. Lesion diameter of ten *Botrytis* isolates 72h after inoculation of detached tomato. (A). Vertical bars indicate standard error ($SE \pm$). Bars with the same letters are not significantly different according to the Duncan's multiple range test ($p \leq 0.05$). (B) Leaf lesions caused by *Botrytis cinerea* (B03) 72 h after plug inoculation.

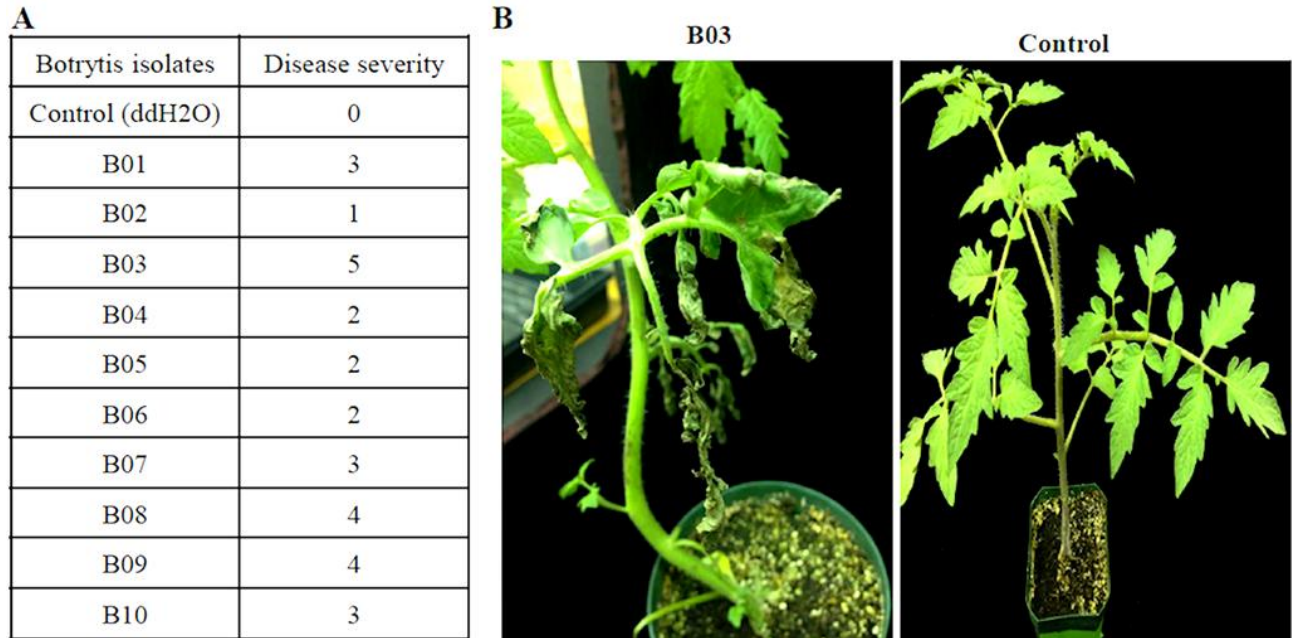


Figure 3.3. A) Disease severity rating of *Botrytis* isolates following spray inoculation onto leaves of tomato (variety Favorite). B) Symptoms caused by *Botrytis cinerea* (B03) two weeks after inoculation; Disease severity rating: (0) no lesions (healthy); small number of lesions (almost healthy); (2) small number of lesions without died leaves; (3) small number of lesions with 1-5 dead leaves; (4) large number of lesions with 6-10 dead leaves; (5) large number of lesions with 11-20 dead leaves.

Inhibitory effect of plant extracts on spore germination

Plant extracts that showed the greatest antifungal activity were from *C. chinense* and *C. annuum*. Spores treated with these extracts at 30 and 40% concentrations showed significant differences compared to spores treated with broth (control) for all absorbance. Treated spores with these extracts registered under 0.1 absorbance in fungal growth after 6, 12, and 24 h (Figure 3.4). An interaction between main treatments and all concentrations was not detected, indicating that the extracts effectively reduced *Botrytis* spore germination at all concentrations. The 40% *C. chinense* and *C. annuum* extracts completely inhibited spore germination and fungal growth of *B. cinerea* after 24 h compared with the control (Figure 3.4). The extracts of *W. indica* and *C. frutescens* had intermediate antifungal activity against spore germination of *B. cinerea* at the 30 and 40% concentrations. The absorbance was less than 0.1 after 6 and 12 h but increased to more than 0.3 with 24h of incubation (Figure 3.4). No effects were observed on spore germination with any of the plant extracts at the 10 or 20% concentrations (Figure 3.4). All plant extracts showed absorbance greater than 0.3 at concentrations of 10 and 20% after 24 h (Figure 3.4). Mycelial growth did not occur when spores were treated with extract concentrations of 40% in *C. chinense* or *C. annuum* after 24 h. However, growth was less in spores treated with the same concentration of *W. indica* and *C. frutescens* extracts compared with the positive and negative control (Figure 3.4).

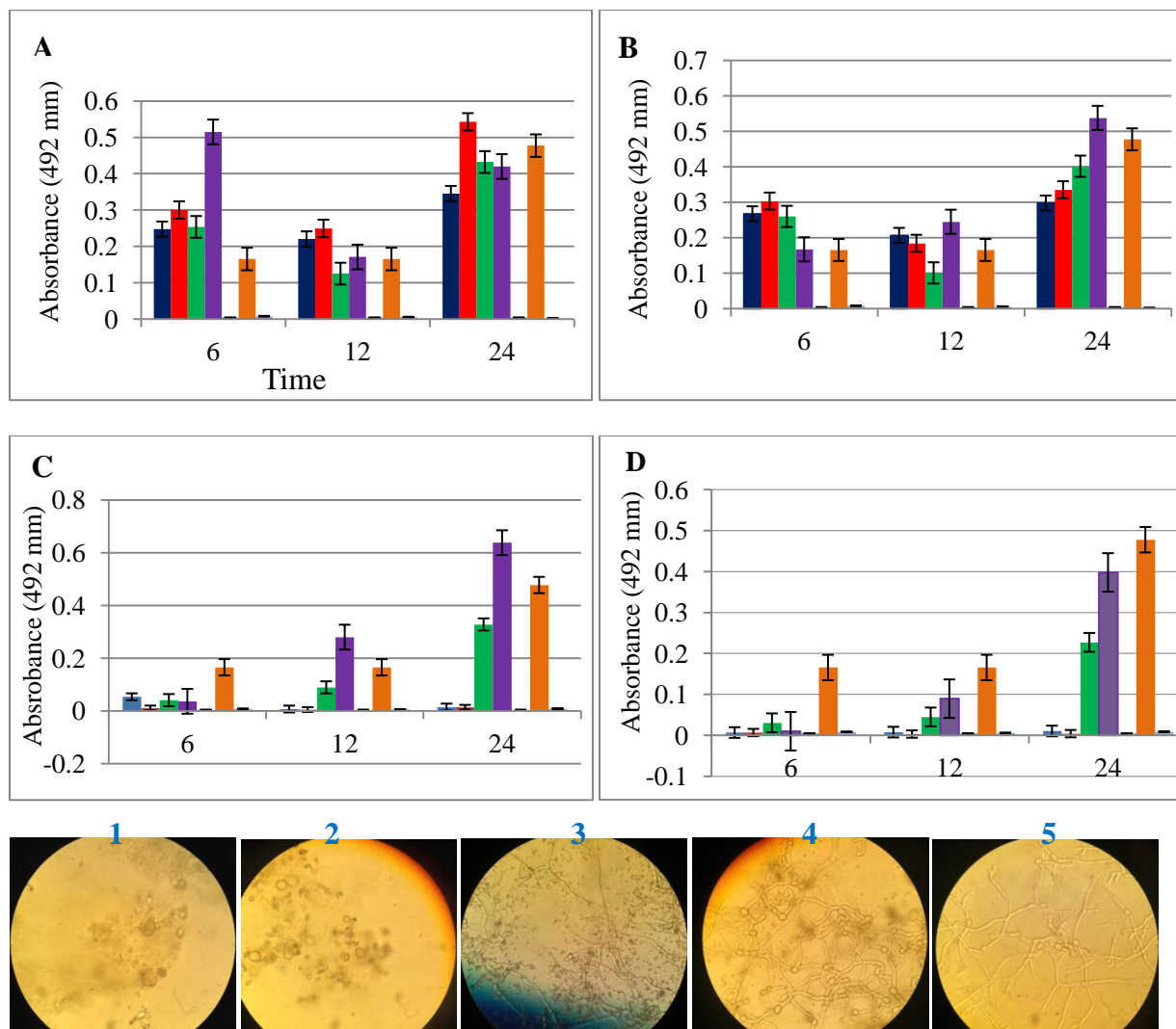
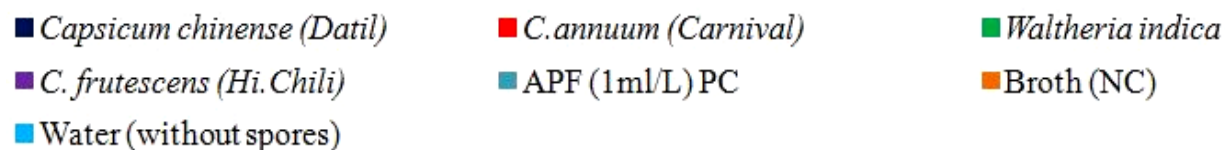


Figure 3.4. Effect of plant extracts on spore germination of *Botrytis cinerea* measured by absorbance of spore suspensions (492 nm) at 6, 12, 24 h. (A) 10% (B) 20% (C) 30% (D) 40% (E) Micrographs of spores treated with 40% extracts after 24h of *Capsicum chinense* (Datil); (2) *C. annuum* (Carnival); (3) *C. frutescens* (Hawaiian Chile pepper); (4) *Waltheria indica*; (5); malt extract broth (control). Vertical bars indicate standard error (SE \pm).

Reactive oxygen species

Reactive oxygen species generation was recorded when *B. cinerea* was exposed to 40% extracts of *C. chinense* cv. Datil and *C. annuum* cv. Carnival for 0, 2, 4, and 6h at 23°C (Figure 3.5). With an increased incubation period, the number of stained spores increased. The highest percentage of stained spores 92% and 66% for *C. chinense* and *C. annuum* extracts respectively. ROS of spores treated with the 40% extracts were greater compared with control treatment where 6% ROS was observed (Figure 3.5). A majority of spores in the control treatment were not stained by DCFH-DA, implying poor ROS production at this time, indicating that an increasing number of oxidizing molecules was produced in spores exposed to the extracts of *C. chinense* and *C. annuum*.

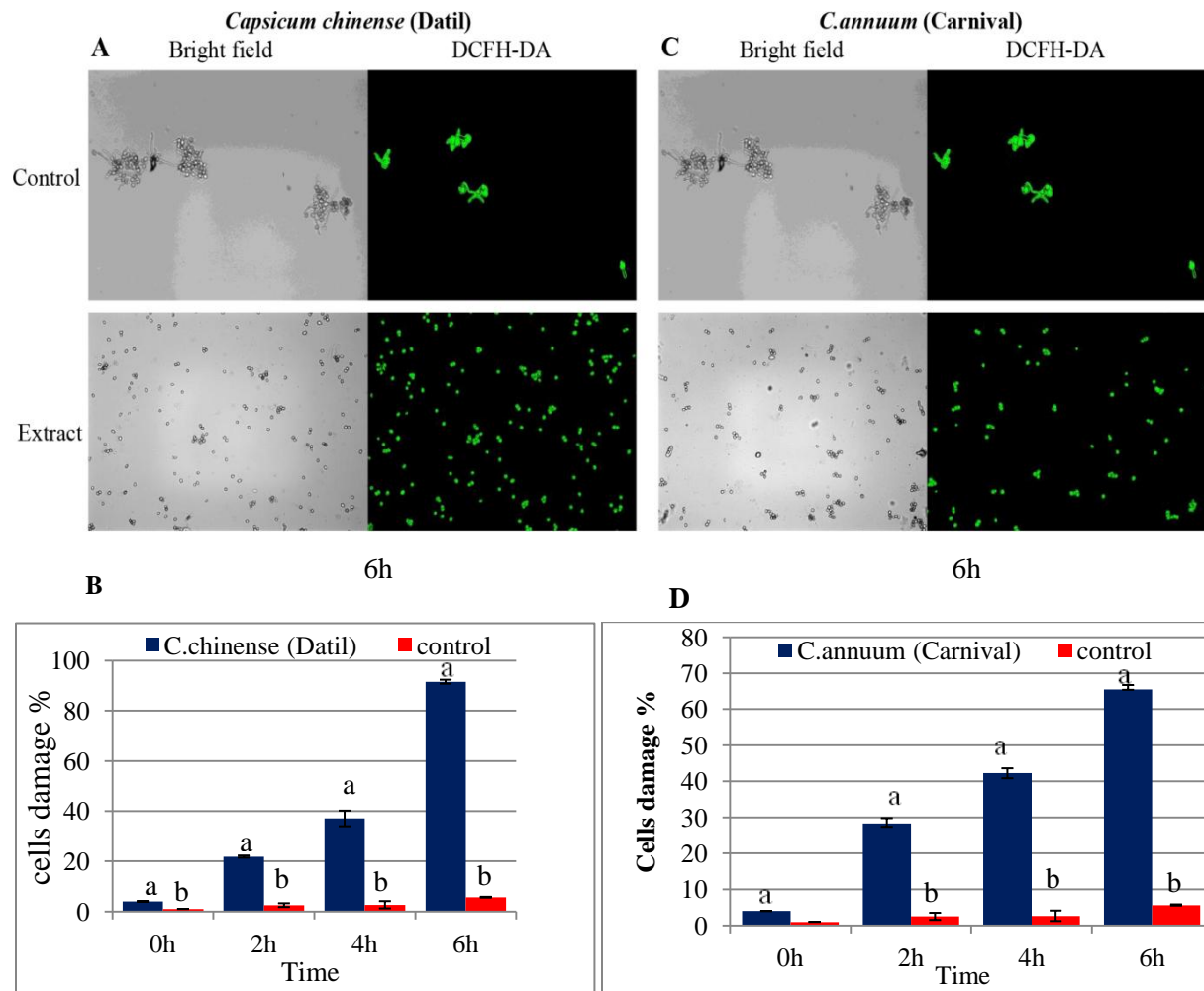


Figure 3.5. Effect of *Capsicum chinense* cv. Datil (A) and *C. annuum* cv. Carnival (B) extracts at 40% on production of reactive oxygen species in spores of *Botrytis cinerea*. (C) and (D) the percentage of spores stained with DCFH-DA. Spores incubated for 0, 2, 4, and 6h. Vertical bars indicate standard error (SE±). Columns followed by different letter are significantly different according to the Duncan's multiple range test ($p < 0.05$).

Plasma membrane integrity

With an increased incubation period, damage to cell membranes increased (Figure 3.6). The highest percentage of the damaged plasma membrane of stained spores was 89 and 51% for *C. chinense* and *C. annuum* extracts respectively following 6h incubation. The control treatment had only 30% damage ($p<0.05$) (Figure 3.6). No difference was observed between treatments and control after 0 and 2 h incubation, indicating that it was necessary to increase the exposure period of spores to the extracts before effects on the cell membrane integrity were observed (Figure 3.6).

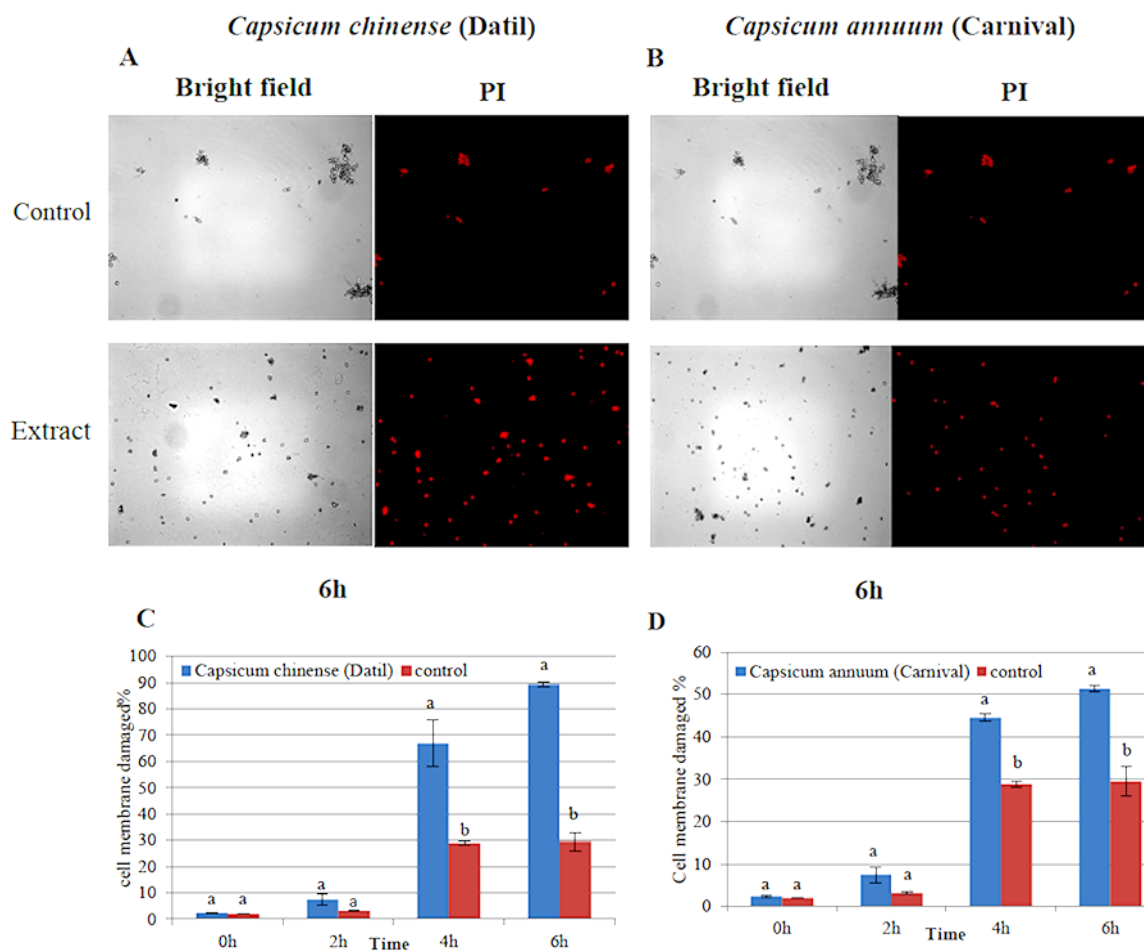


Figure 3.6. Effect of *Capsicum chinense* cv. Datil (A) and *C. annuum* cv. Carnival (B) extracts at 40% on cell membrane in spores of *Botrytis cinerea*. The percentage of cell membrane damage of spores treated with a 40% extract of *C. chinense* cv. Datil (C) or *C. annuum* cv. Carnival (D) and stained with propidium iodide. Spores were incubated for 0, 2, 4, and 6h. Vertical bars indicate standard error ($SE \pm$). Bars with the same letter are not different according to the Duncan's multiple range test ($p < 0.05$).

Effect of plant extracts on fungal spores analyzed by TEM

Transmission electronic microscopy was used to visualize morphological changes in *B.cinerea* spores treated with 40% of *Capsicum chinenses* and *C. annuum* extracts individually for 2 and 6 h at $25\pm 2^{\circ}\text{C}$. Intact cell walls, cell membranes and cytoplasm were normal showing no obvious changes in morphological structure in the untreated control sample of *Botrytis cinerea* spore (Figure 3.7 A & D). However, fungal grown in nutrient broth supplemented with 40% plant extracts showed distinct signs of deterioration, deformation or condensed cytoplasm after 2 h of exposure to either *C. chinense* (Figure 3.7 B) or *C. annuum* (Figure 3.7 E). Areas of dense cytoplasm became larger upon six hour exposure of fungal spores to plant extracts of either *C. chinense* (Figure 3.7 C) or *C.annuum* (Figure 3.7 F) whereas there were no areas of dense cytoplasm in the germinated spores of the control (Figure 3.7 D). Organells of fungal spore treated with *C. chinense* for 2 h were obviously disrupted (Figure 3.7 E).

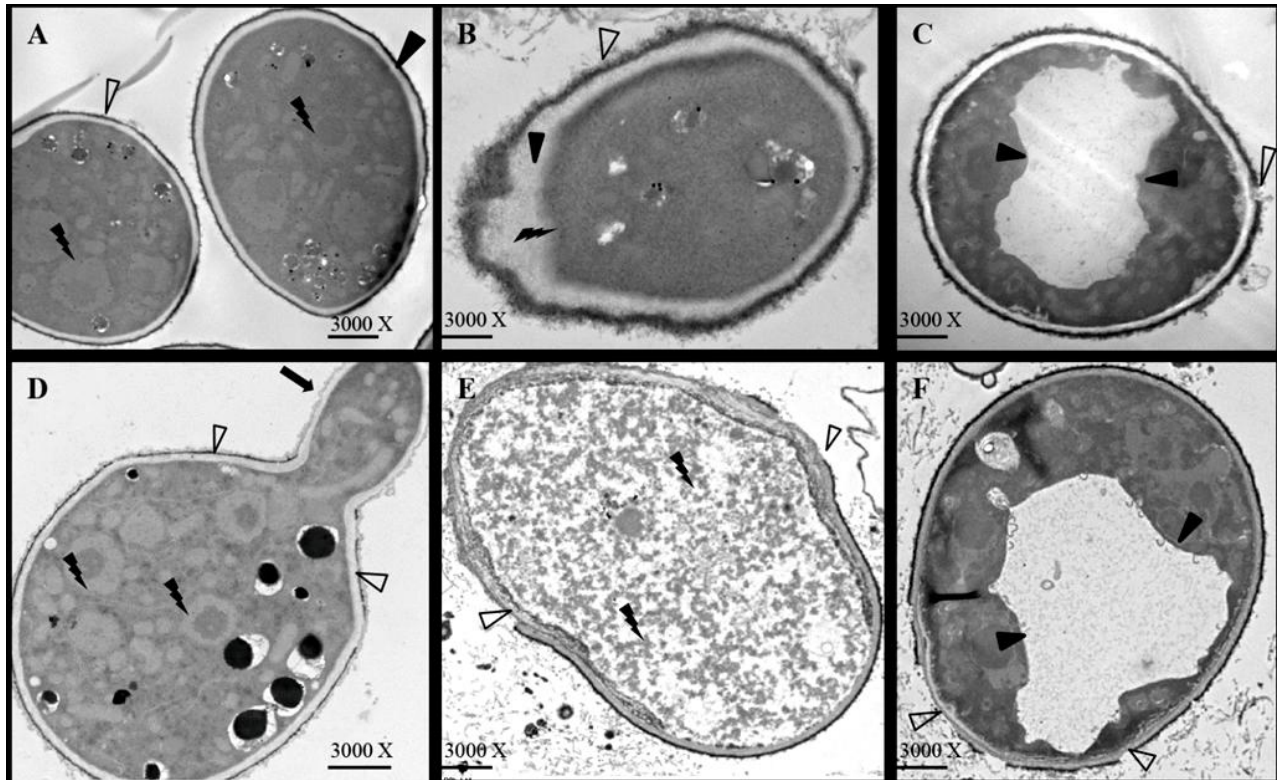


Figure 3.7. Micrographs of *Botrytis cinerea* spores examined with transmission electron microscopy. Spores were incubated in potato dextrose broth (PD) without *Capsicum chinenses* Datil and *C. annuum* extracts (A,D) respectively or with 40% the extracts for 2 h (B, E) and 6 h (C, F). In the absence of plant extracts, the cytoplasm (symbols with black lightning bolt), cell wall (clear arrows) and cell membranes (black arrows) were intact and appeared normal. Following treatment with 40% plant extracts the cell walls and membranes were degraded, the cytoplasm was condensed and cell structure was abnormal.

Effect of *Capsicum chinense* and *C.annuum* extracts on gray mold disease of tomato fruit

As a curative treatment, both plant extracts were not effective in reducing gray mold disease ($p > 0.05$). As a preventive treatment, however, *C. chinense* applications at 40 and 60 % completely inhibited lesion development following immersion for 10 min (Figure 3.8 & 3.10). In contrast, *C.annuum* applications at 60 % inhibited lesion development after 10 min immersion (Figure 3.9 & 3.10) Fruits dipped into 60% of *C. chinense* for 10 min prior to inoculation differed in lesion diameters after 7 days incubation at 23°C compared with the control or treatments at lower rates ($p < 0.05$) (Figure 3.8). These results were consistent with data from spore germination, reactive oxygen species and plasma membrane integrity. The results indicate that *C. chinense* and *C. annum* extracts reduced postharvest gray mold of tomato fruit when fruit were immersed in 60% plant extracts for 10 minutes.

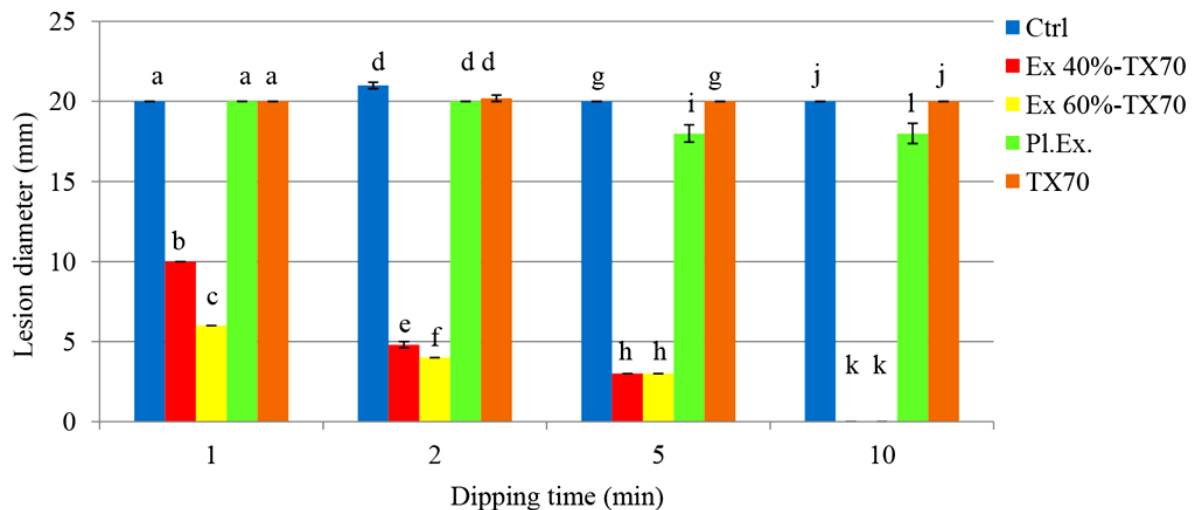


Figure 3.8. Effect of different concentrations of *Capsicum chinense* cv. Datil extract at different immersion times on disease severity in tomato fruit following inoculation with *Botrytis cinerea*. The mean lesion diameter was measured after the treated fruit were stored at 25°C for 7 days. Mean values showing different letters are statistically different according to Duncan's multiple range test ($p < 0.05$).

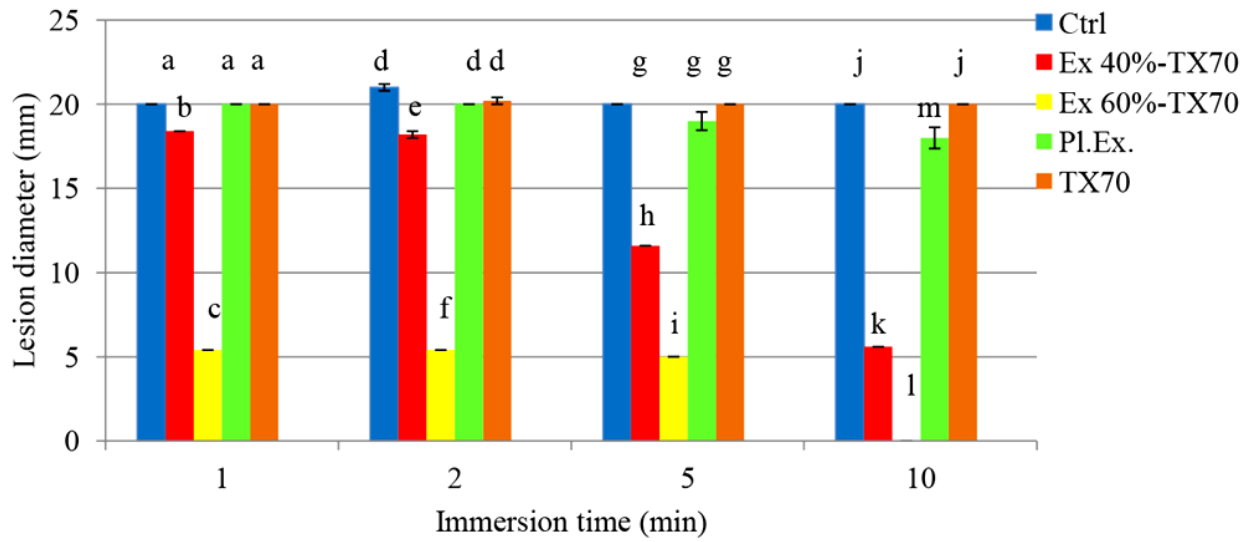


Figure 3.9. Effect of different concentrations of *C. annuum* cv. Carnival extract at different immersion times on disease severity in tomato fruit following inoculation with *Botrytis cinerea*. The mean lesion diameter was measured after the treated fruit were stored at 4°C for 7 days. Mean values showing different letters are statistically different according to Duncan's multiple range test ($p < 0.05$).

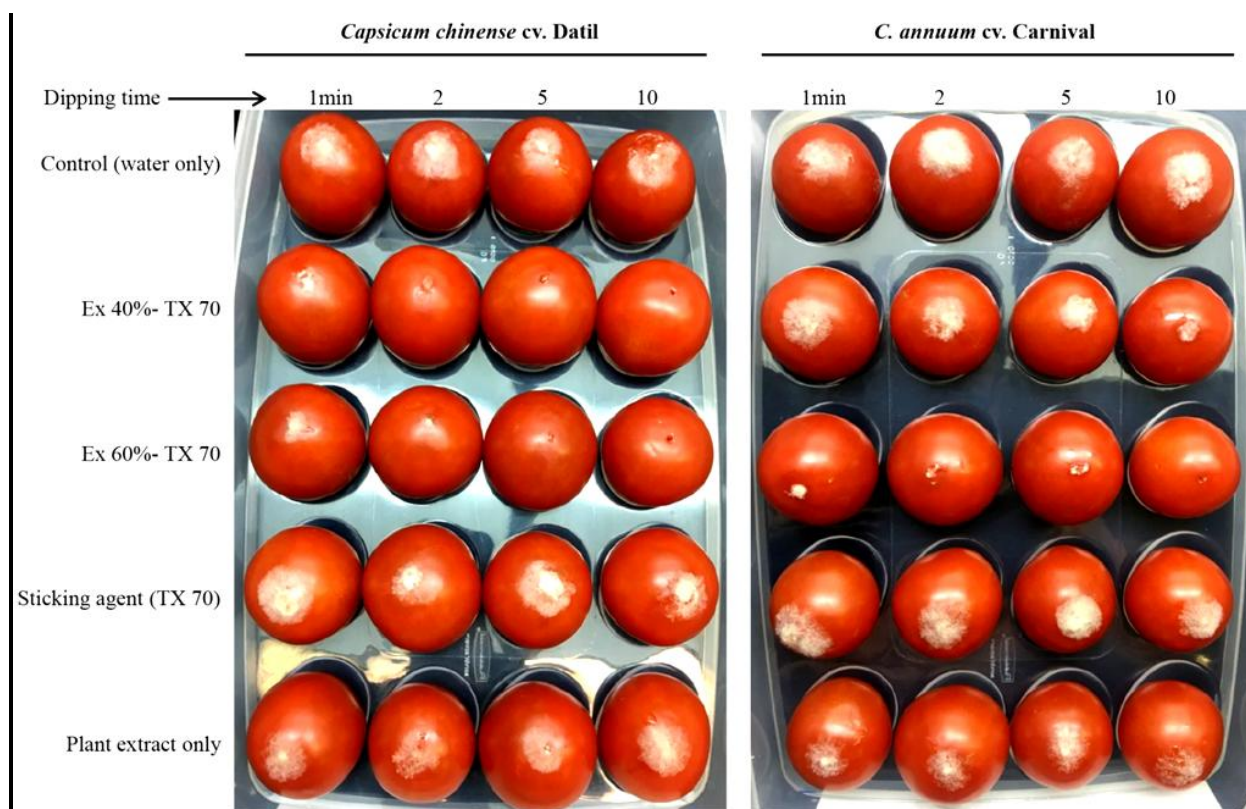


Figure 3.10. Photograph showing the efficacy of plant extracts (A) *Capsicum chinense* and (B) *C. annuum* at 40 and 60% mixed with a sticky agent on disease severity on tomato fruit with varying concentrations and immersion times. Fruit was treated 10 min prior to inoculation with *Botrytis cinerea* (B03). Inoculated fruit were stored for 7 days at 28°C then evaluated. The photographs were taken seven days after inoculation.

Discussion

The high variability in severity of symptoms caused by *Botrytis* isolates on tomato fruit, detached leaves, and whole plant suggests the existence of wide pathogenic variability among the isolates. Pathogenic behavior of isolates is frequently a result of deviation in cell wall degrading enzymes (CWDEs), activities and existence of oxalic acid and secreted pathogenicity factors (Bellincampi et al., 2014; Kubicek et al., 2014). High concentration of oxalic acid is associated with high levels of pathogen virulence (Kumari et al., 2014; Mbengue et al., 2016). Some studies demonstrated that the variation in *Botrytis* isolates was in their secreted polygalacturonases and pectin lyases and this contributed to the pathogenicity range of pathogens (Cotoras and Silva, 2005). Other studies showed the pathogenic behavior of isolates is associated with the dispersal of a transposable element that contributes to pathogenicity and disease severity (Fournier et al., 2005; Samuel et al., 2012).

Many natural fungicides occur in plants and can serve as alternatives to synthetic fungicides. Pesticide resistance is a major problem when applying fungicides such as Fenhexamid against *Botrytis cinerea*, (De Miccolis Angelini et al., 2014; Hahn, 2014).

Most of the plant species utilized in this experiment are edible and traditionally used in medicinal recipes (Everitt and Drawe, 1999; Zongo et al., 2013). Among them, *Capsicum chinense* (Datil) and *C. annuum* (Carnival) extracts showed high antifungal activity followed by *C. frutescens* and *Waltheria indica*. Extracts from *Capsicum* species were demonstrated to have greater antifungal activity against *Botrytis cinerea* (Wilson et al., 1997). The antifungal activity of *Capsicum* extract could be attributed to capsaicin, phenolic compounds, flavonoids, steroids, alkaloids, and tanins (Gayathri et al., 2016). Although these extracts have been recognized as possessing antifungal compounds, they have not been developed into commercial products for

postharvest treatments because industries utilize available synthetic products and patenting new products are costly and time- consuming. *C. chinense* and *C. annuum* extracts inhibited spore germination of *B. cinerea*. *Capsicum* extracts have potential as antifungal agents as well as alternatives to synthetic fungicides for postharvest fungal infections. Capsicum extracts could be used as pre- and/or postharvest applications for protection of tomato fruit from gray mold disease.

The mechanism of action associated with extracts of *Capsicum* seems to be related to production of ROS. Cellular dysfunction or cell death is a consequence of oxidative damage to cellular components caused by ROS (Shi et al., 2012; Wu et al., 2014; Wu et al., 2016). ROS was induced by the Capsicum extract and inhibited spore germination of *B. cinerea*. PI staining suggested that the accumulation of ROS in spores that led to cell membrane damage or reduced the membrane integrity. Moreover, knowledge of the mode of action of Capsicum extracts on *B. cinerea* would be useful for pre- and postharvest management against gray mold. Consequently, further studies are needed on effects of Capsicum extracts not only against pathogenic fungi but also against pathogenic bacteria that can also cause postharvest diseases on fruit and vegetables such as *Pectobacterium* sp.

Conclusion

Botrytis isolates differed in their virulence that was shown during the pathogenicity tests. Also, we conclude that *C. chinense* cv. Datil and *C. annuum* cv. Carnival extracts at concentrations of 40% inhibited spore germination of *B. cinerea*. In addition, Capsicum extracts induce ROS generation in fungal spores, leading to oxidation and cell membrane damage. The oxidative damage could account for the effectiveness of Capsicum extracts against *B. cinerea*. Preventive ten- minute dip applications of either *C. chinense* cv. Datil or *C. annuum* cv. Carnival

extracts at 60% reduced the lesion diameter in fruit inoculated with *Botrytis cinerea*. These two natural products could be alternatives to synthetic fungicides for control of postharvest gray mold in tomato.

CHAPTER IV

ANTIBACTERIAL EFFECT OF POTASSIUM TETRABORATE ON PECTOBACTERIUM CAROTOVORUM IN TOMATO

Introduction

Tomato is an important economic crop worldwide and soft rot disease caused by *Pectobacterium carotovorum* leads to significant postharvest losses. Soft rot is a predominant progressive decay characterized by tissue maceration, such that the entire fruit is damaged (Bartz and Wei, 2002; Charkowski, 2006). Bacterial infections occur in the field, transit, packing and/or during storage. Quantitative losses caused by soft rot are greater than any other bacterial disease (Bhat et al., 2010). The bacteria are not able to penetrate the surface of tissue directly but rather enter through wounds and natural openings. The bacteria then multiply in the intercellular spaces, where they produce pectolytic enzymes and degrade cell middle lamellae leading to softening of infected tissue (Yang et al., 1992). Currently, there are few effective ways to decontaminate infected tomato fruit or prevent cross- contamination that do not pose concerns to human health; thus, disease management essentially depends on cultural practices such as avoiding over-irrigation, maintaining proper harvesting, handling and packing practices, cleaning and disinfestation of harvesting equipment, and proper storage conditions (Gnanamanickam, 2006; Jones et al., 2014).

Studies going back to the 1920's have shown the efficiency of inorganic salts, such as aluminum chloride, sodium thiosulfate, and sodium benzoate to control postharvest diseases such as soft rot of potato (Yaganza et al., 2014), gray mold of grapevines and tomato (Jeandet et al., 2000; Wu et al., 2015), decay of melons (Aharoni et al., 1997) and citrus green mold (Smilanick

et al., 1999). Potassium tetraborate tetrahydrate ($B_4K_2O_7 \cdot 4H_2O$) (PTB) is one of the inorganic salts used for preservation wood worldwide (Freeman et al., 2009). Some studies demonstrated the effectiveness of PTB application to control common postharvest fungal diseases, such as gray mold caused by *Botrytis cinerea* in table grapes (Qin et al., 2010), anthracnose caused by *Colletotrichum gloeosporioides* in mango (Shi et al., 2011; Shi et al., 2012). PTB has been used in industry and agriculture as safe compounds for control of many fungi and insects (Qin et al., 2007). However, there is no documentation on the use of PTB to control postharvest diseases caused by bacteria. Flow cytometry is a rapid sensitive method for quantifying live and dead cells in suspension (O'Brien-Simpson et al., 2016; Massicotte et al., 2017). Propidium monoazide (PMA) is a high-affinity photoreactive DNA binding dye that has recently been used to distinguish intact from membrane- damaged bacterial cells and other microorganisms. PMA only penetrates dead bacterial cells with damaged membranes but not live cells with the intact membranes (Nocker et al., 2006). PMA combined with qPCR was successfully used to quantify the viability of bacterial cells (Bae and Wuertz, 2009; Kim and Ko, 2012). The objective of this study was to investigate the effectiveness of PTB for reducing soft rot of tomato caused by *P. carotovorum*.

Materials and Methods

Bacterial strain

The bacterial strain used for these studies was *P.carotovorum* BA17, which was the most virulent strain isolated in Hawaii. Pathogenicity was confirmed on three types of tomato including, common market, cherry and grape tomato. Several bacteriological tests including oxidation/fermentation (OF), production of catalase, degradation of sodium polypectate, KOH test and hydrolysis of esculin and starch were conducted on each strain. Presumptive

identifications were confirmed by 16S rDNA sequence analysis (Ahmed et al., 2017a). All strains were maintained in freezers at -80°C for further testing.

Determination of antibacterial activity and minimum bactericidal concentration of PTB in vitro conditions

The antibacterial activity of PTB (Sigma Aldrich, St. Louis, MO, USA) was assessed for *P. carotovorum* using a paper disk diffusion method (Jorgensen and Turnidge, 2015). One ml of an overnight broth culture of bacteria was diluted to obtain an inoculum of 10^8 CFU/ml using a spectrophotometer (600 nm). A 50 μ l aliquot was spread evenly on solid agar plates containing Luria media using sterile swabs. Sterile 13-mm paper disks were saturated with PTB in increasing concentrations ranging from 10 to 120 mM. The first tests were run at pH 9.2 (with no pH adjustment) and a second set was run after adjustment to pH 7.0 following neutralization with 0.1N HCl. Plates were divided into four quarters, and one paper disk was placed in each quadrant. Paper disks saturated with sterile water were used as controls. Plates were incubated at 28°C for 24 hours, and the inhibition zone area was measured. For determination of minimum bactericidal concentration (MBC), tubes containing Luria broth and PTB at concentrations ranging from 10 to 100 mM were inoculated with 20 μ l aliquots of a *P. carotovorum* suspension that contained 10^8 CFU/ml. Tubes were placed in the incubator shaker for 16 h at 28°C, 140 rpm. Two controls were used: i) inoculated Luria broth lacking PTB, and ii) non-inoculated broth. After incubation, Aliquots (20 μ l) of resuspended media were spotted onto Luria agar plates divided into four quadrants. Plates were incubated at 28°C for 24 hours. Plates were checked daily over the next 7 days to determine whether growth occurred. The lowest concentration of PTB that killed the *P. carotovorum* was recorded as MBC. The experiments were performed with four replicates and repeated three times. To confirm that the effect was bactericidal and not bacteriostatic, the experiment above was repeated except that broth cultures

were centrifuged, the pellet containing bacterial cells were washed three times with sterile saline, and resuspended in Luria broth lacking PTB. Aliquots were again spotted onto Luria agar plates and incubated as above. This experiment was repeated twice.

Validation of antimicrobial activity of PTB using flow cytometry analyses

Flow cytometry was performed to validate the bactericidal activity of PTB using viability dyes, Syto9 (membrane permeable) and propidium iodide (PI, membrane impermeable) (O'Brien-Simpson et al., 2016). Control samples of live (measured by colony plate counts) and dead cells (killed by 70% isopropanol) were first established as controls for flow cytometry runs before determining the numbers of live and dead cells in samples treated with PTB. Liquid NB 24 h old cultures of *P.carotovorum* were treated with different concentrations of PTB (50-, 60-, 70-, 80-, 90-, and 100 mM) for 16 h at 28°C with shaking at 140 rpm. The bacterial suspensions were centrifuged, and the pellet was washed twice with 0.85% NaCl. Bacterial cells were stained with the LIVE/DEAD *BacLight* kit components of Syto9 and propidium iodide (Thermo Fisher, Grand Island, NY, USA) following the protocol provided by the manufacturer. Samples were analyzed with a Beckman-Coulter EPICS XL flow cytometry instrument (FlowJo, Ashland, OR) using 15 mW 488 nm excitation (argon ion laser). The fluorescence signals from the Syto9-stained cells (Kantor et al.) were collected (green 525 nm BP filter), along with PI-stained cells (DEAD) (red 610 nm BP filter). Forward and side light scatter signals were measured. Two-parameter histograms of log PI fluorescence vs. log Syto9 fluorescence were analyzed in FlowJo software designed to distinguish live and dead cells and to calculate the percentage of each cell type.. Live and dead cells clustered in two separate sections of dot blots. A diagonal line was drawn to separate the two clusters and a gate was drawn to establish borders of each blot.

Numbers of cells within each gate were counted by the cytometry. Each experiment was conducted with four replicates, and the test was repeated three times.

Validation of antimicrobial activity of PTB using qPCR-based method with propidium monoazide (PMA) to distinguish viable from nonviable bacteria cells

A PMA assay was conducted using the BLU-V Viability PMA kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. A suspension of *P. carotovorum* containing 10^8 CFU/ml was treated with 100 mM PTB in nutrient broth for 16 hours at 28°C with shaking (140 rpm). Four control samples were prepared: live and dead bacterial cells, each with and without PMA. As a control, bacterial cells were killed by heating for 10 min at 70°C in a thermomixer. Treated and nontreated bacterial suspensions were centrifuged for 5 min at 13,000 g. A 10 µl volume of 2.5 mM of PMA reagent was added to the cell pellet containing 500 µl of EB buffer. After incubation for 10 min at room temperature in the dark, samples were exposed to light for 20 min using a 650-W halogen light source (Kobayashi et al., 2009). The samples were laid on the ice at a 45 angle and 20 cm in front of the light source. After photoactivation of PMA, the bacterial suspension was centrifuged at 7,600 rpm for 10 min, and DNA was extracted.

DNA extraction and SYBR Green Real-time qPCR

A DNeasy Blood & Tissue Kit (Qiagen) was used to isolate DNA from PMA-treated and nontreated *P. carotovorum* cultures following the manufacturer's protocol. Primer set PEC-F (5'-GTGCAAGCGTTAATCGGAATG-3') and PEC-R (5'-CTCTACAAGACTCTAGCCTGTCAGTTT-3') targeting the 16S rRNA gene (Pritchard et al., 2013) was used for SYBR Green qPCR-based specific detection of *P. carotovorum*; primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). SYBR Green qPCR assays were performed in a 25 µl reaction mixture containing 12.5 µl SsoAdvance Universal Supermix (Bio-Rad, Hercules, CA,

USA), 8.5 µl of ultrapure water (Thermo Fisher Scientific, Grand Island, NY, USA), and 0.2 µM of each forward and reverse primer. SYBR Green qPCR conditions were 30 s at 95°C followed by 30 cycles of 10 s at 95°C and 30 s at 60°C using CFX96 Real-Time PCR Detection System (Bio-Rad). Three replicates were used for each reaction and water was used as a non-template control. Cycle threshold was set manually, and Bio-Rad CFX Manager 3.1 software was used to analyze the data.

Transmission electron microscopy

Damage to bacterial membranes treated with and without PTB was assessed using TEM following the method of Al-Adham et al., (2000) with slight modifications. An overnight culture *P. carotovorum* (BA17) was grown on NB supplemented with or without 100 mM PTB for 16 h at 28°C with shaking 140 rpm. One-ml aliquots were centrifuged at 4000 x *g* for 10 min at room temperature and washed twice with sterile distilled water. The pelleted cells were fixed with 4% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2, held at room temperature overnight and washed in 0.1M cacodylate buffer 2 x for 10 min, followed by postfixation with 1% OsO₄ in 0.1M cacodylate buffer for 1h. Cells were dehydrated in a graded ethanol series (30, 50, 70, 85, 95, and 100%), substituted with propylene oxide, and embedded in LX112 epoxy resin and placed in a 60°C oven for 24h to polymerize the resin. Ultrathin (60-80 nm) sections made on a resin-fixed pellet using RMC PowerTome Ultramicrotome and double stained with uranyl acetate and lead citrate. Sections were examined utilizing a Hitachi HT7700 TEM (Hitachi, Tokyo, Japan) at 100 kV and photographed with an AMT XR-41B 2k x and 2k CCD camera.

In vivo effects of PTB on soft rot disease symptoms on tomato fruit

Protective action of PTB

A method using potassium tetraborate tetrahydrate was used to assess the efficacy of PTB to protect the tomato fruit from soft rot caused by *P. carotovorum*. Tomato fruit that were uniform in size and color, free from wounds and rot were selected. The fruit were washed with tap water; surface sterilized by dipping in 1% sodium hypochlorite solution for 10 min, rinsed by immersing in two changes of sterile distilled water, and dried in ambient air. A wound 1 mm diameter in 4 mm deep was made on each fruit using a pipette tip. Wounded fruit were immersed for 10 min in the different PTB concentrations (0-, 90-, 100-, and 120- mM) for 1-, 2-, 5- and 10-min, and then dried in ambient air. Each wound was inoculated with a 10- μ l aliquot containing 1×10^8 CFU/ml of *P. carotovorum* (Chen and Zhu, 2011). Bacterial suspensions were prepared from 24 h-old cultures grown on Luria agar (Difco, Sparks, MD, USA). Fruit were placed onto wet paper towels in a plastic container and incubated for 3 days at 24°C. Disease severity was assessed by measuring lesion diameter on each fruit. Each treatment contains five fruit. Lesion diameter was the mean of the five fruit in each replicate. The experiment was repeated twice.

Curative action of PTB

A curative assay was conducted using the procedures used for the protective assay except that the wounded fruit were immersed in the borate solution for 12 h after inoculation (Yaganza et al., 2014). Each treatment was replicated by five fruit and the test was repeated twice.

Statistical analysis

The experiments were set up as Complete Randomized Design (CRD) with four replications. Data were analyzed using SAS 9.2 V (SAS Institute Inc., Cary, NC, USA) and

means were compared by Duncan's multiple range tests. Differences at $p < 0.05$ were considered significant. The experiments were repeated two to three times.

Results

Molecular identification

A PCR product obtained for all bacterial strains was the expected size (1.4 kb). An NCBI BLAST showed that the sequences of BA12 and BA15 had $> 98\%$ similarity to *Pectobacterium* spp. For BA17, the similarity was $\geq 100\%$ to *P. carotovorum*.

Antimicrobial activity of PTB at pH 7.0 and pH 9.2

Maximum inhibition of *P. carotovorum* occurred at 100 mM. The inhibition zone increased with the increase of PTB concentrations ($p < 0.05$). At 100 mM PTB, the inhibition zone was 22 mm compared with control (water only) 0.00 mm regardless of the pH (Figures 4.1, 4.2). There was no difference in the inhibition zone at 100 and 120 mM PTB ($p > 0.05$). The results confirm that while the high pH (pH 9.5) accounts for some bactericidal activity, PTB also had bactericidal activity.

Minimum bactericidal concentration (MBC)

The number of colonies that grew on test plates decreased as the PTB concentrations in the medium increased (Figure 4.3). No bacterial colonies appeared on plates containing 100 mM PTB after 24 h incubation at 28°C, and there was no regrowth on these plates after 7 days. Furthermore, after washing pelleted cells three times before plating onto Luria agar, no colonies grew from tubes containing 100 mM PTB, and this was established as the MBC.

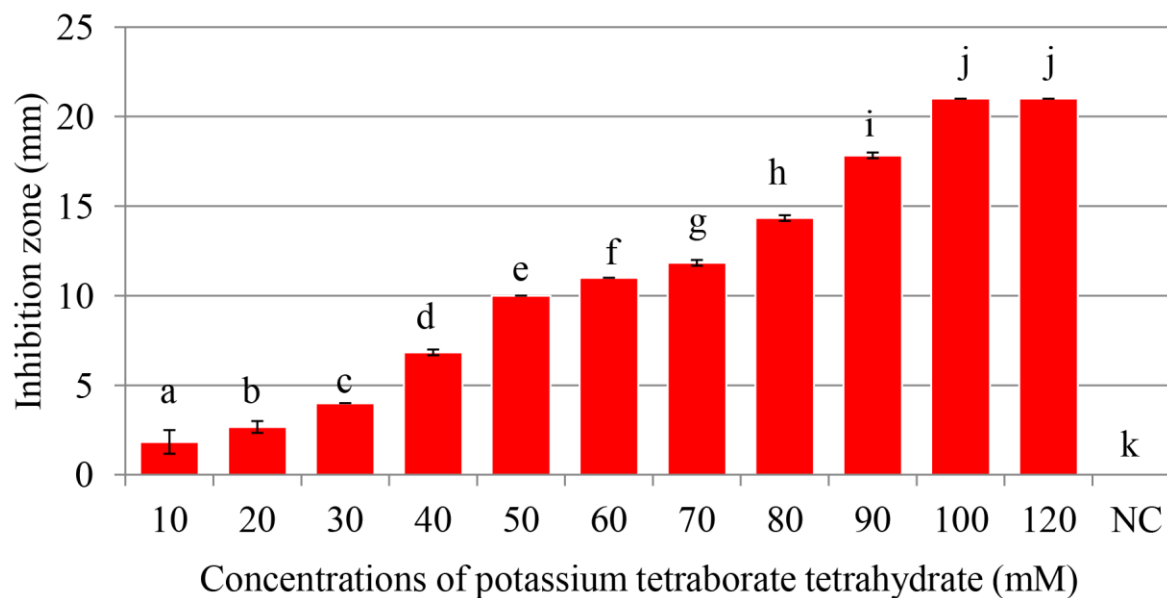


Figure 4.1. Antibacterial activity of potassium tetraborate tetrahydrate (PTB) at pH 9.2. Maximum inhibition of *Pectobacterium carotovorum* occurred at 100 mM. Vertical bars show mean values and standard error (\pm SE). Means with different letters are statistically different according to Duncan's multiple range test ($p < 0.05$).

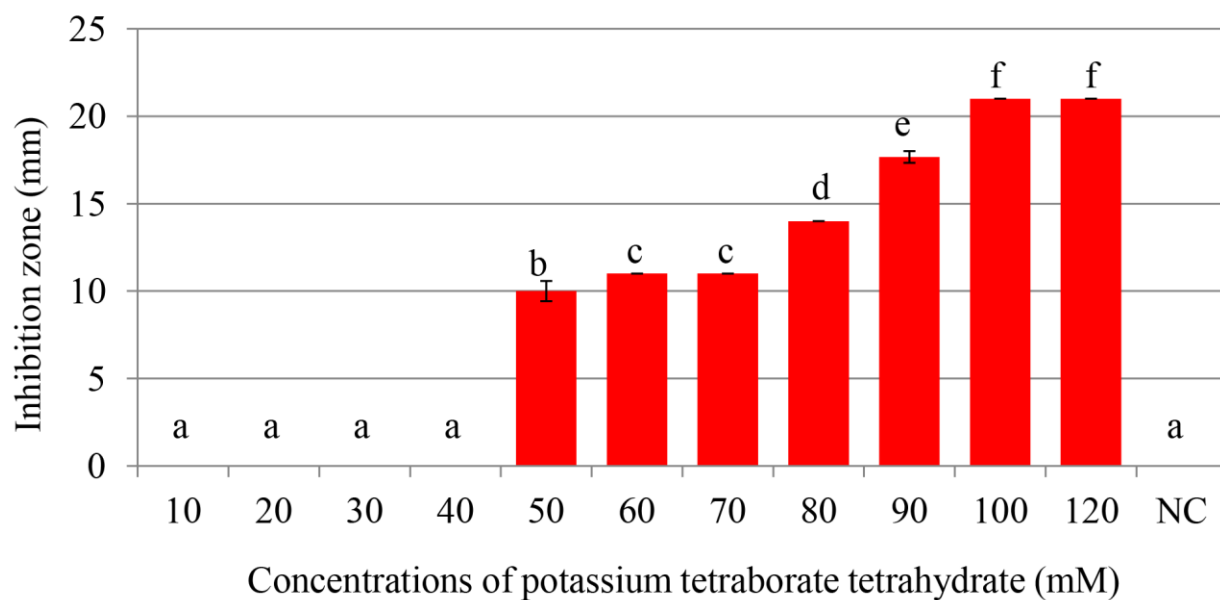


Figure 4.2. Inhibition of *Pectobacterium carotovorum* by increasing concentrations of potassium tetraborate tetrahydrate (PTB) at pH 7.0. Bacterial growth was partially inhibited at 50 mM; maximum inhibition zones were produced at 100 mM PTB. Vertical bars show mean values and standard error (\pm SE). Means with different letters are statistically different according to Duncan's multiple range test ($p < 0.05$).

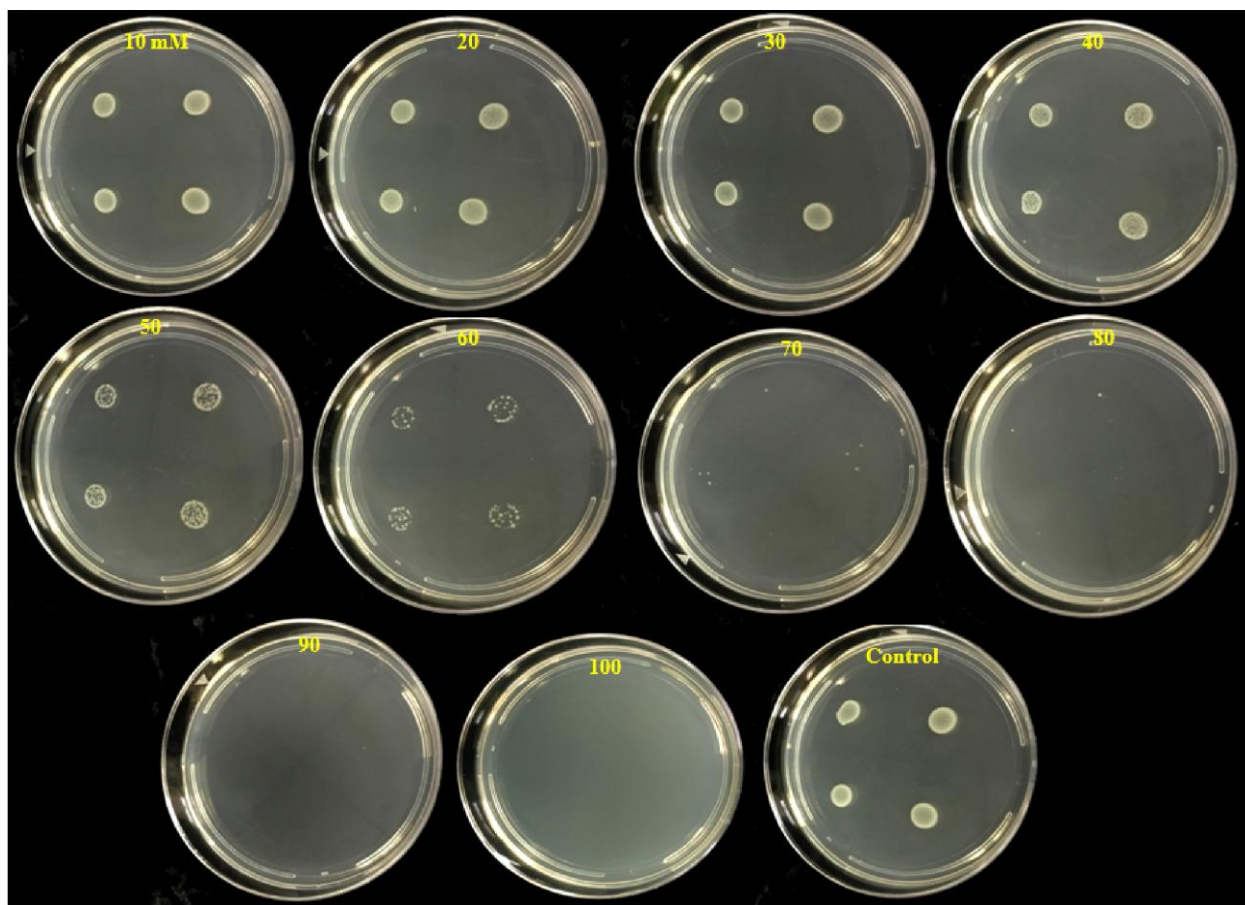


Figure 4.3. Inhibition of colony development of *Pectobacterium carotovorum* by increasing concentrations of potassium tetraborate tetrahydrate (PTB). Aliquots (10 μ l) containing 10^7 CFU of *P. carotovorum* were deposited at four sites on a series of Petri plates which contained different concentrations ranging from 10 to 100 mM PTB. Two colonies developed on plates containing 80 mM, one colony was visible on the plates containing 90 mM and no colonies developed on plates containing 100 mM PTB.

Flow cytometric analysis of bactericidal activity of PTB

Live cells with intact cell membranes were distinguished from dead isopropanol-treated bacterial cells as shown on flow cytometry dot blots and histograms (Figure 4.4). Live cells of *P. carotovorum* stained with Syto9 dye and 98% of the data points were below the diagonal line whereas 2% of the PI stained cells were dead and were above the diagonal line (Figure 4.4 A). Conversely, with the dead-cell-control, 97% of the data points were in the upper gate whereas 3% of the data points were in the lower gate (Figure 4.4 B). Following treatment with 50 mM PTB 56% of the cells were dead and 44% were live (Figure 4.4 C). The percentage of dead cells in the upper gate continued to increase as the PTB concentrations increased (Figure 4.4 D & H). The non-treated cells stained with Syto9 dye and 95% clustered in the lower gate and gave a histogram similar to the live cell-control.

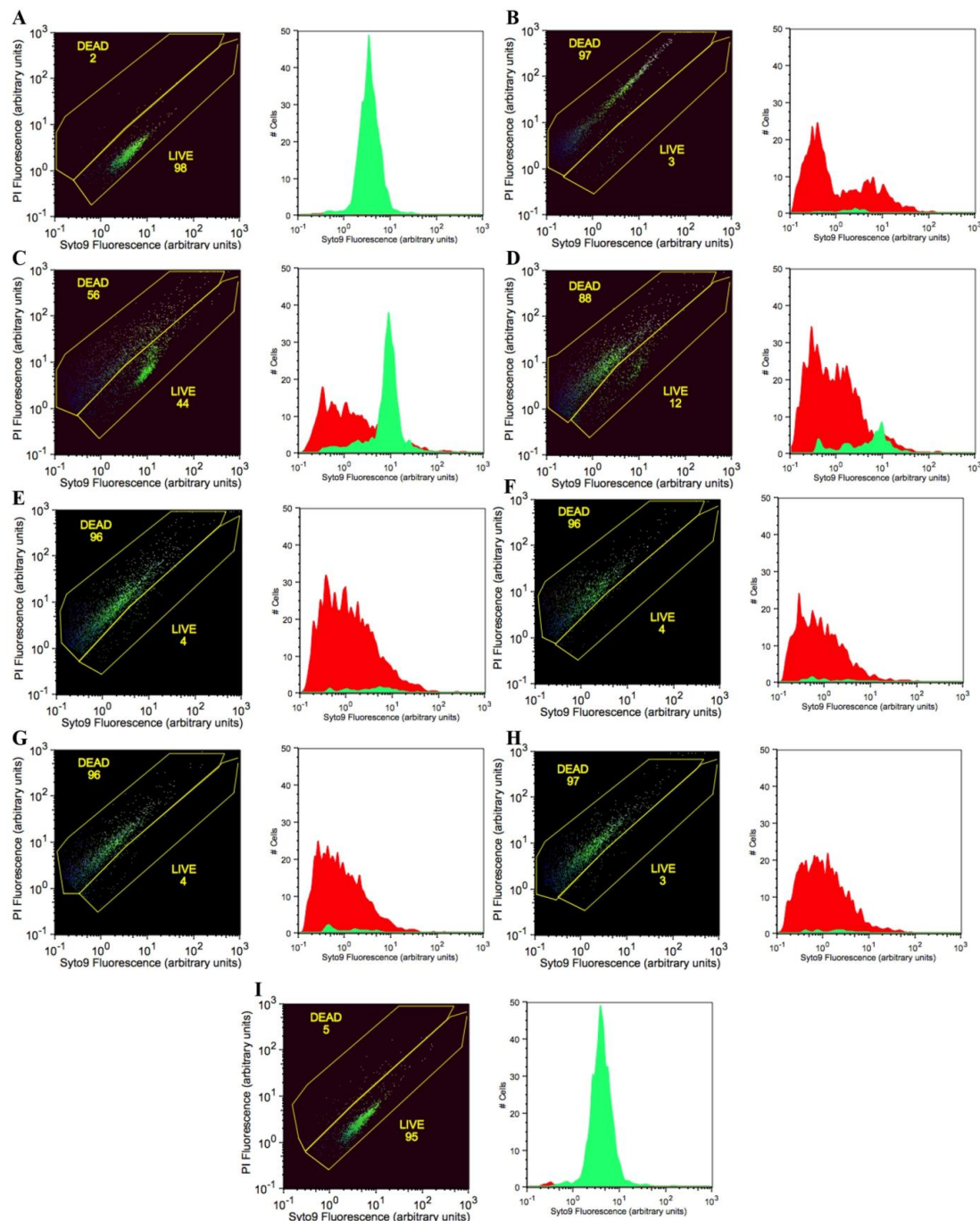


Figure 4.4. Flow cytometry dot blots and histograms which differentiate live from dead cells. Increasing concentrations of potassium tetraborate tetrahydrate (PTB) increases bacterial membrane permeability to propidium iodide as shown by flow cytometry with Syto9 and PI staining. (A) live cells (non-treated; calibration standard); (B) dead cells (killed with 70% isopropanol; calibration standard). Bacterial cells treated with PTB at increasing millimolar concentrations (C) 50; (D) 60; (E) 70; (F) 80; (G) 90; (H) 100; (I) Non-treated bacteria (control). The X-axis indicates the intensity of Syto9 green fluorescence and the Y-axis indicates the intensity of PI red fluorescence (arbitrary units: a). flow cytometric data are represented as the percentage of live and dead cells (dot blot) with the fluorescence Syto9 and PI dyes. The diagonal line in the dot blot separates dead cells (above the line) from live cells (below the line) and the borders of the cell clusters are established as the gate. Graphs show the images present the cell counts of live (green) and dead (red) cells.

Real-time qPCR with PMA treatment for analysis of bactericidal activity of PTB

Quantitative PCR was performed as an additional validation method for confirming bactericidal activity of PTB at 100 mM. When PMA was absent from bacterial suspensions, qPCR did not distinguish between live and dead cells (Figure 4.5). Addition of PMA to live bacterial suspensions did not affect the cycle threshold (Ct) values because PMA cannot penetrate membranes of intact cells and had no effect on qPCR. In contrast, when PMA penetrates the membranes of dead bacterial cells, it binds to the DNA and delays amplification, which results in higher Ct values for the sample. Both the heat- and PTB-treated cells had significantly higher Ct values (25) than live cells (Ct value, 15). The results obtained by PMA-qPCR coincided with results of flow cytometry and culture plate assays confirming complete kill of bacterial cells with 100 mM PTB.

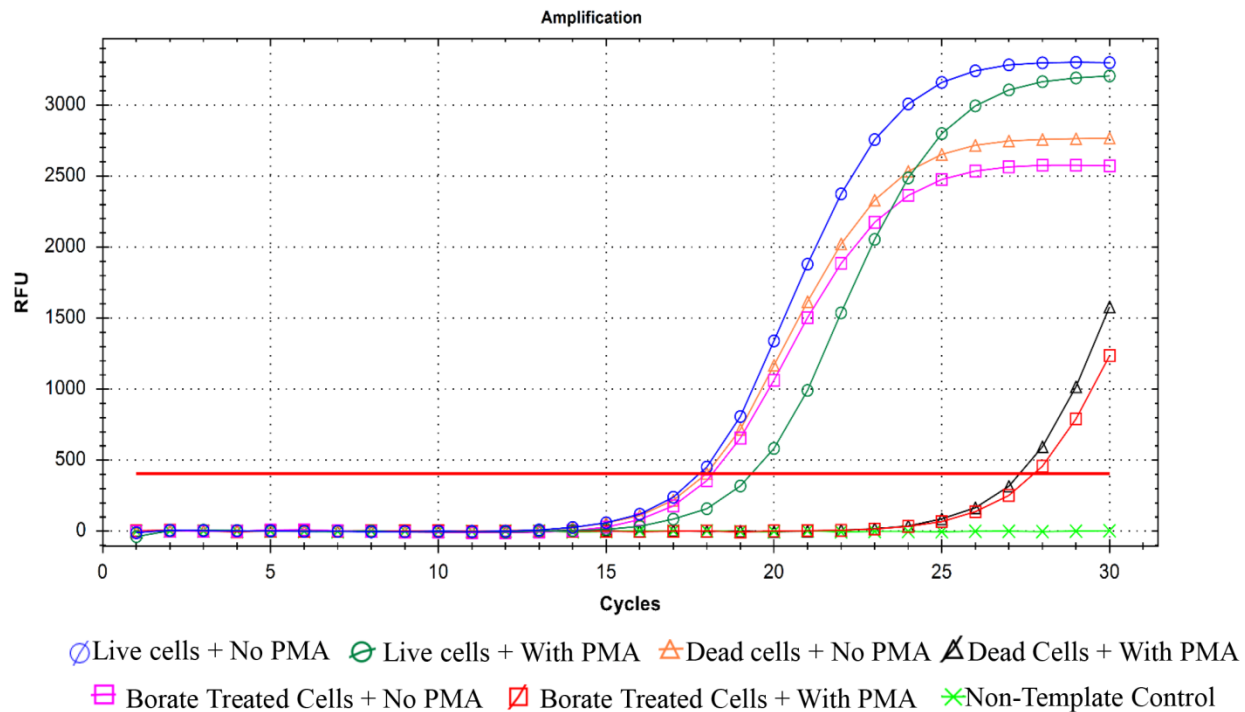


Figure 4.5. Quantification real-time PCR for *Pectobacterium carotovorum* treated with 100 mM potassium tetraborate tetrahydrate (PTB) with and without propidium monoazide (PMA). Data are averages of three separate experiments.

Effect of PTB on ultrastructure of bacterial cell analyzed by TEM

Transmission electronic microscopy was used to visualize morphological changes in *P. carotovorum* cells treated with 100 mM PTB. Cell membranes and cytoplasm were normal showing no noticeable changes in morphological structure in the absence of PTB (Figure 4.6 A). The cells in the untreated control sample of *P. carotovorum* showed intact cell membranes with no deformation or dense cytoplasm. In contrast, bacterial cells grown in nutrient broth supplemented with 100 mM PTB showed distinct signs of deterioration (Figure 4.6 B). The cell membrane was distorted and the cytoplasm exuded from membranes of all the cells observed.

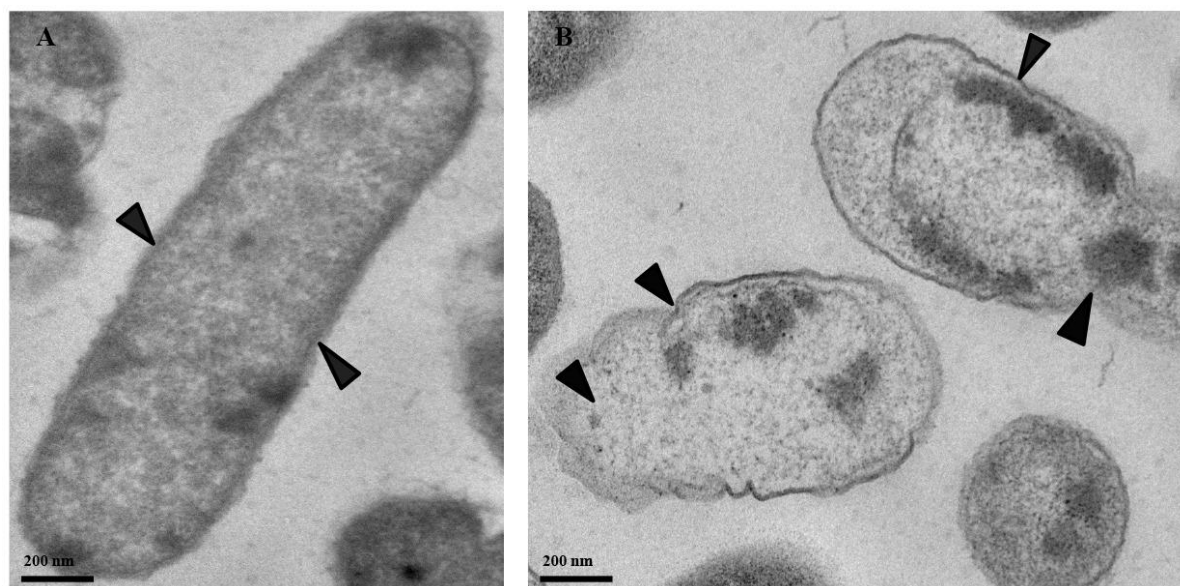


Figure 4.6. Micrographs of *Pectobacterium carotovorum* cells examined with transmission electron microscopy. Bacteria were incubated in nutrient broth medium (NB) without potassium tetraborate tetrahydrate (PTB) (A) or with 100 mM PTB (B). In the absence of borate, the cytoplasm and cell membranes were intact and appeared normal (A; black arrows). Following treatment with 100 mM PTB the cell membranes were degraded, the cytoplasm exuded from damaged areas and cell structure was abnormal (B; black arrows).

Effect of PTB on soft rot disease of tomato fruit

PTB was not effective as a curative treatment for reducing soft rot disease ($p > 0.05$). As a preventive treatment, PTB applications at 100 and 120 mM completely inhibited lesion development (Figure 4.7 & 4.8). Fruit dipped into PTB (100 and 120 mM) for 5 and 10 minutes prior to inoculation differed in lesion diameters after 7 days incubation at 23°C compared with the control or treatments at lower rates ($p < 0.05$). (Figure 4.7). These results were consistent with data from MBC, flow cytometry, and qPCR tests. The results indicate that PTB reduced postharvest soft rot of tomato fruit when fruit were immersed in 100 mM PTB for 5 minutes. Lesion development was completely inhibited following emersion for 10 minutes.

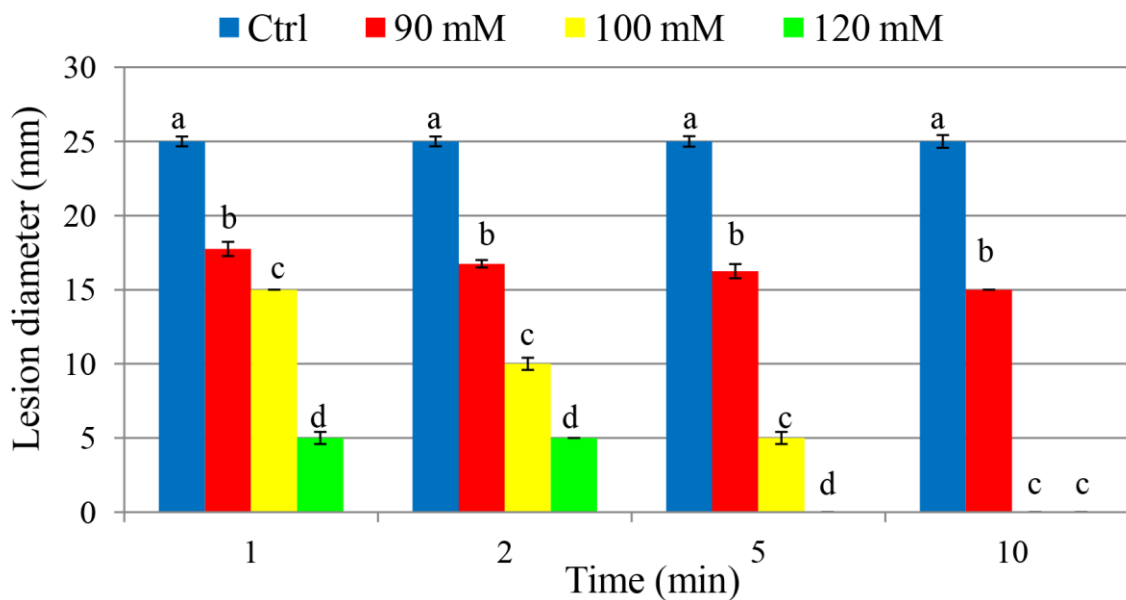


Figure 4.7. Effect of different potassium tetraborate tetrahydrate (PTB) concentrations at different immersion times on disease severity in tomato fruit following inoculation with *Pectobacterium carotovorum*. The mean lesion diameter was measured after the treated fruit were stored at 28°C for 7 days. Mean values showing different letters are statistically different according to Duncan's multiple range test ($p < 0.05$).

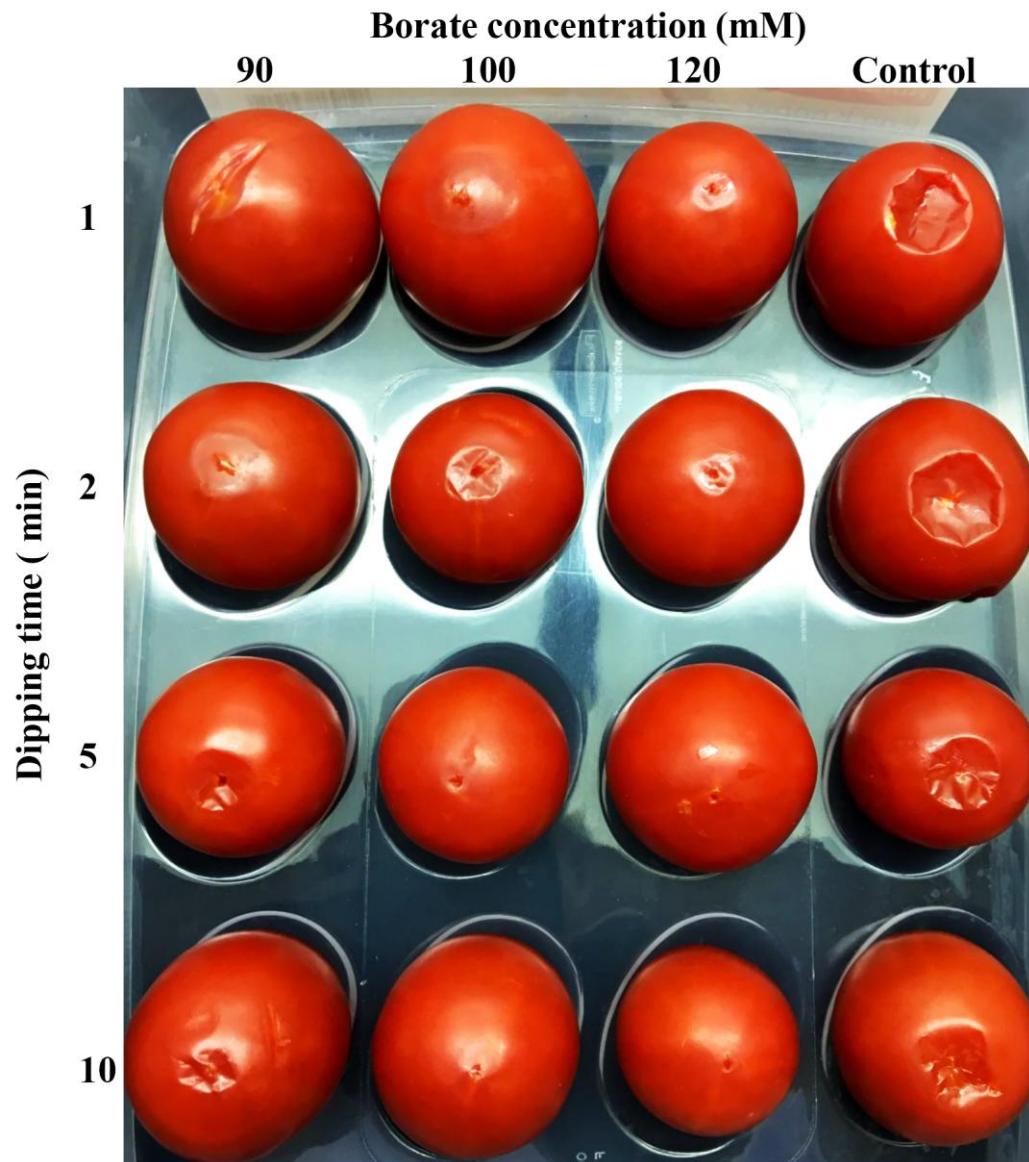


Figure 4.8. Photograph showing efficacy of potassium tetraborate (PTB) on disease severity on tomato fruit with varying concentrations and immersion times. Fruit were treated 10 min prior to inoculation with *Pectobacterium carotovorum*. Inoculated fruit were stored for 7 days at 28°C then evaluated. The photograph was taken seven days after inoculation.

Discussion

Boron is an essential microelement for plant growth and enhancement of fruit quality. In tomato adequate levels of boron are needed to avoid ruptures in the cuticle (Strong and Robert, 2001; Huang and Snapp, 2009). Recently, several studies have demonstrated that PTB can be used as an antifungal compound for the control of postharvest diseases (Qin et al., 2010; Thomidis and Exadaktylou, 2010; Shi et al., 2011; Shi et al., 2012). Other studies have shown that boron (Kartal et al., 2004) and PTB (Reeb, 1997) reduced wood damage by termites and fungi. Our study was the first to evaluate the potential of PTB for postharvest management of soft rot bacterial disease caused by *P. carotovorum*. PTB at 100 mM completely inhibited the growth of *P. carotovorum*, both at its normally high pH (9.5) and after neutralizing to pH 7.0. Salt solutions of PTB, potassium carbonate, sodium bicarbonate and sodium carbonate also exhibited antifungal and antibacterial effects independently of pH (Nigro et al., 2006; Qin et al., 2010). Thus, the antibacterial activity of PTB was not due solely to disruption of cell membranes at high pH. The inhibitory effect of boron (as borax and boric acid) on *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Actinetobacter septicus* was similar with and without adjusting the pH value (Yilmaz, 2012).

In the past, Arab physicians used borate as cleaning agents and internal medications (Kingma, 1958). Borate possesses microbiostatic and microbiocidal effects and is also used in medicinal applications including wet dressings (Fisher, 1956), preservation of urine samples (Watson and Duerden, 1977) and medical eye lenses (Houlsby et al., 1986).

Several possible mechanisms for the inhibitory effect of borate include binding of borate ions to chemical energy transporters such as ATP, NAD, and NADH leading to impaired protein synthesis, disruption of mitochondria and prevention of cell division (Kim et al., 2003; Reid et

al., 2004). Excessively high salt concentrations disrupt osmoregulatory processes and reduce bacterial growth. High alkalinity can denature proteases on the cell surface, change the cytoplasmic pH and disrupt DNA (Gould, 1991). The water-ionizing capacity and the lipophilicity of inorganic salt components play an important role in the inhibition of bacterial growth (Yaganza et al., 2009). In addition to water-ionizing capacity, borate salt is hydrophobic enabling the borate salt to interact with lipid components of the bacterial cell membrane. This interaction leads to dysfunction of the bacteria and results in growth inhibition. Furthermore, borate inhibits enzymatic reactions such as hydrolase, transferase, dehydrogenases, and oxidoreductase (Johnson and Smith, 1976; Hunt, 1996). Nicotinamide adenine dinucleotide (NAD) is an important coenzyme in all living cells for metabolism and electrons transfer. Boron binding affinity has been demonstrated for many ribose-comprising nucleotides and cofactor such as adenosine phosphates groups (ATP, ADP, AMP), NADH, NAD, cAMP, and SAM (Ralston and Hunt, 2001). Electrospray ionization mass spectrometry showed that borate binds to both *cis*-2, 3-ribose diols on NAD⁺ forming esters and only monoesters formed with NADH. The esterification of borate with NAD and NADH occurred under alkaline conditions at pH 7.0 to 9.0 (Kim et al., 2003). Some studies suggest that borate alters the equilibrium constant of any reaction containing *cis*-diols and competes with enzymes for NAD⁺ (Smith and Johnson, 1976). Therefore, direct interaction between borate and nucleotides is one of the possible inhibitory mechanisms for the antibacterial activity of borate.

The bactericidal activity of PTB against *P. carotovorum*, was confirmed in our study by flow cytometry using a protocol to assess bactericidal activity (O'Brien-Simpson et al., 2016). Increased PI staining of *P. carotovorum* cells treated with PTB indicates cell membrane disruption resulting in a bactericidal rather than a bacteriostatic effect. The relationship between

the borate concentration and the percentage of dead cells was generally linear. The analysis by flow cytometry confirmed results observed in the cultural antibacterial assays. No bacterial colonies developed following a 10-minute treatment with PTB at 100 mM or higher. As a further validation, we used quantitative PCR with propidium monoazide, a DNA binding agent, which can only penetrate cells with severely damaged membranes. PMA has been used in combination with quantitative PCR to differentiate intact from dead bacterial cells (Kobayashi et al., 2009; Desfossés-Foucault et al., 2012). The Ct value of borate-treated bacteria was similar to the Ct value of the dead-cell control, indicating that PTB destroyed cell membranes permitting the PMA to mask DNA and make the maximum primer binding sites unavailable. These results confirm previous results from the cultural and flow cytometry tests. Transmission electron microscopy showed degradation of bacterial cell membranes, exudation of cytoplasm and abnormal cell structure, indicating that treated cells were damaged by PTB. Antifungal action of PTB also was associated with damage to *Colletotrichum gloeosporioides* (Shi et al., 2011; Shi et al., 2012) and *Botrytis cinerea* (Qin et al., 2010). *Pectobacterium atrosepticum* cells were rapidly damaged and killed by exposure to sodium metabisulfite and aluminum chloride salts (Yaganza et al., 2004).

As a preventative treatment, PTB applications showed a significant reduction in the development of soft rot disease of tomato fruit. However, it had no curative action, most likely because bacterial multiplication cannot be arrested once the bacteria enter wounded fruit. PTB is a promising bactericidal agent for controlling soft rot disease caused by *P. carotovorum* and presents low risk to human health (Çelikezen et al., 2014). In addition, application of PTB is inexpensive and can be applied to tomato fruit before shipping to the market.

Conclusion

PTB salt at 100 mM was bactericidal against *P. carotovorum* and its bactericidal activity was confirmed by TEM, flow cytometry and qPCR. A preventive one- to five-minute dip in potassium tetraborate at 100 mM significantly reduced lesion diameters in fruit inoculated with *P. carotovorum*. No lesions developed following a 10-minute dip treatment. PTB can be a safe and cost-effective alternative for preventing soft rot disease in postharvest tomato fruit.

CHAPTER V

REDUCTION OF GRAY MOLD WITH PREHARVEST APPLICATIONS OF POTASSIUM TETRABORATE AND ANOTHER PROPRIETARY FORMULATION ON GREENHOUSE TOMATO

Introduction

Gray mold, caused by *Botrytis cinerea*, is one of the most destructive diseases of greenhouse tomatoes and causes massive losses in tomato fruit during pre- and postharvest period (Elad et al., 2007; Williamson et al., 2007; Jones et al., 2014). *B. cinerea* is second among fungal pathogens of economic significance (Dean et al., 2012). In 2006, approximately 344 hectares of greenhouse tomatoes were grown in the United States (Ingram and Meister, 2006) and currently greenhouse tomatoes account for 50 to 73% of all fresh market production at retail grocery stores and large outlets, respectively, in the United States (Michael Bledsoe, *personal communication*). *B. cinerea* is the most common pathogen of greenhouse tomato, which is highly susceptible to this necrotrophic fungus (Jones et al., 2014). Humidity of 90% or greater plays a major role in development of gray mold in the greenhouse (Jarvis, 1989). Moreover, tomatoes are highly perishable and have a short shelf life due to susceptibility to mechanical damage and infection by other plant pathogens before or after harvest (Coates and Johnson, 1997). Postharvest fungal decay occurs at different stages during the retail chain: shipping, storage, display, customer selection, and consumption (Williamson et al., 2007). Synthetic fungicides such as fenhexamid and benzimidazol are still used to minimize postharvest diseases. Other fungicides, such as dichloran have restricted use in greenhouse tomato for gray mold control in the United States (Ingram and Meister, 2006). The capacity of fungal pathogens to develop

resistance to fungicides and public concern about potentially harmful impacts of fungicides on human health (Hahn, 2014; Romanazzi et al., 2016) have promoted exploration of alternative environmentally safe fungicides. Several studies have established the efficacy of potassium tetraborate (PTB) in controlling gray mold using postharvest applications on table grapes (Qin et al., 2010), mango rot caused by *Colletotrichum gloeosporioides* (Shi et al., 2012), and brown rot caused by *Monilinia laxa* on peaches (Thomidis and Exadaktylou, 2010). In a previous study, a ten min immersion of tomato fruit in potassium tetraborate tetrahydrate PTB reduced the incidence of soft rot caused by *Pectobacterium carotovorum*. (Ahmed et al., 2017b). Mycelial growth of *B. cinerea* was inhibited at 1 ml/L of an Agrichem Proprietary Formulation (APF) (Ahmed et al., 2016) and spores were killed *in vitro* at the same concentration (Ahmed et al., 2017b). The objective of the current study was to determine whether PTB or APF would be effective in preharvest applications for managing postharvest gray mold disease on tomato.

Materials and Methods

Assays of antifungal activity

Ten *Botrytis* isolates collected from infected tomatoes in a previous survey were tested for sensitivity to PTB as previously tested with APF (Agrichem, Inc., Australia). A single *B. cinerea* isolate, B03, was selected for greenhouse studies because it was the most virulent isolate when tested on three tomato varieties, common, grape and cherry tomato (Ahmed et al., 2017a) and was sensitive to APF. A culture medium (V8) was autoclaved and allowed to cool at 45°C and supplemented with PTB at a final concentration of 25 mM. A plug of *Botrytis* mycelium (6 mm diameter) was transferred to a 100-cm Petri plate containing V8 and PTB. Plates were incubated at 25°C, and mycelial growth was measured when growth in the control plate reached the edge of the Petri dish.

Phytotoxicity of potassium tetraborate (PTP) and the Agrichem proprietary formulation (APF) on cherry tomato plants

Phytotoxicity assays for PTB and APF were conducted on 5-week-old tomato plants (var. Kewalo). Plants were sprayed individually with solutions of 25-, 50-, 75 mM PTB or APF at 1-, 2-, and 4 ml/L to runoff. Control plants were sprayed with water. Phytotoxicity on leaves (discoloration, burn and necrosis) was recorded after 10 days.

Selecting the variety of cherry tomato

Several varieties of cherry, grape and common tomato varieties were evaluated for yield and fruit size in order to select the most appropriate variety for subsequent evaluations of disease control measures. Experiments were initiated on June 5, 2015 at the Pope Environmental Laboratory/greenhouse located at the University of Hawaii Manoa. Four tomato varieties (Skuar (F1), Favorite (F1), Jasper, and Supersweet) were purchased from (Johnny's Selected Seeds Company, Winslow, USA) and two (Grape tomato, and Kewalo varieties) were provided by the UH Manoa Seed Laboratory. Seeds of these varieties were germinated in 10-cm diameter plastic pots containing Sunshine Mix No.4 and pots were placed on the greenhouse bench. Three seedlings were transplanted after 2 weeks into (10 inch x 9 inch depth) plastic pots filled with Sunshine Mix No.4. Pots were arranged on greenhouse benches equipped with trellises. Plants were fertilized with 14-14-14 (NH₄: P₂O₅: K₂O) every 4 weeks and then with soluble fertilizer Miracle-Gro (at the rate of 2.6 g/L of water) every two weeks. The experiment had six varieties, five replicates for each variety and two plants per replicate. Data recorded for each variety included: number of days for seed germination, days to fruit set and the number of fruit.

Preharvest Spray Trials

Preparing plants, inoculation and preharvest spray with APF and PTB

B. cinerea B03 was stored at 4°C in sterilized soil and revived for inoculum production on 9-cm diameter Petri dishes containing V-8 agar. The plates were incubated for 10 to 14 d at 23°C with a 12-h photoperiod (Carisse and Van Der Heyden, 2015). Spore suspensions containing 1×10^5 spores/ml were prepared from 10-d-old cultures using a hemocytometer. Experiments were initiated on March 6, 2017 at the Pope Environmental Laboratory/greenhouse located at the University of Hawaii Manoa (Figure.5.1). Seeds of cherry tomato (Favorite F1) were germinated in 10-cm diameter plastic pots containing Sunshine Mix No.4 and pots were placed on the greenhouse bench. Three seedlings were transplanted after 2 weeks into (10 inch x 9 inch depth) plastic pots filled with Sunshine Mix No.4. Pots were arranged on the greenhouse benches equipped with trellises. Plants were fertilized with 14-14-14 (% NH₄: P₂O₅: K₂O) every 4 weeks and then with soluble fertilizer Miracle-Gro (at the rate of 2.6 g/L of water) every two weeks. The experimental design had nine treatments, four replicates for each treatment.

Table 5.1. Mean values of days of seed germination, days of blooming flowers, days of fruit set, number of fruits/plant for six tomato varieties in the greenhouse assessed during June, 2015.

Variety	Seeding date	Days for seed germination	Days to the first bloom	Days to fruit set	Number of fruit/plant ^a
Sakuar (F1)	06/09/2015	9	42	50	102
Jasper	06/09/2015	9	42	50	112
Favorite (F1)	06/18/2015	7	33	41	266
Supersweet	06/18/2015	7	33	41	109
Grape tomato	06/09/2015	9	43	51	113
Kewalo	06/09/2015	9	55	70	70

^a Five plants were used to calculate the average of fruit per plant



Figure 5.1. Set up for inoculation of tomato plants in greenhouse experiments. Set A) Inoculation chamber; B) Tomato fruits were bagged for 72 h following spray with spores of *Botrytis cinerea*; C) Lesions developed on fruit 5 days after inoculation; D) Healthy fruit of cherry tomato ‘Favorite’ at harvest.

Tomato plants were inoculated with 1×10^5 spores of *B. cinerea*/ml at fruit set, approximately 5 weeks after transplant. Six fruit clusters (with at least 15 tomatoes/cluster) were tagged per plant and inoculated with a suspension of *B.cinerea* containing 10 mM sucrose and 10 mM monopotassium phosphate (Muckenschnabel et al., 2002) to facilitate the spore adhesion. Fruit clusters were sprayed to run off at 5:30 pm using a hand sprayer. Fruit were covered with a plastic bag sealed to maintain humidity at 95%, and maintained 23°C for 72 h. Plants with inoculated clusters were covered with 75% sun screen shade-fabric. A portable box fan was

placed 50 cm below each bench to maintain the temperature around the inoculated plants at $20\pm5^{\circ}\text{C}$. Tomato clusters were sprayed to run-off with PTB (25 mM) and APF (1 ml/L) using a hand sprayer at tomato ripening stages of turning, pink, and light red (USDA-ERS, 2005). Distilled water was sprayed as a control. Non-inoculated plants were separated from other plants in a different section in the greenhouse to eliminate cross contamination with *Botrytis* spores. The fruit were harvested after they reached the final (red) ripening stage, placed in a plastic bag and maintained at 25°C or 4°C . After 10 days, lesions were counted (50 fruit per replicate) and the percentage showing *Botrytis* lesions was recorded.

Statistical analysis

The experiments were set up as factorial in Complete Randomized Design (CRD) with four replications (Table 1). Data were analyzed using (SAS 9.2 V.USA) and means were compared by Duncan's multiple range test. Differences at $P < 0.05$ were considered significant. The experiments were conducted twice.

Table 5.2. Experimental design for evaluating Agrichem Proprietary Formulation (APF) and potassium tetraborate (PTB) applied at three different ripening stages of tomato fruits.

Tomato ripening stage		Treatments	
Turning	APF	PTB	Control (water only)
Pink	APF	PTB	Control
Light red	APF	PTB	Control

Results

Inhibitory effect of PTB on fungal colonies

Ten *B. cinerea* isolates showed no mycelial growth on media containing 25 mM PTB. Spores on control plates containing no PTB germinated and mycelium grew normally (Figure 5.2).

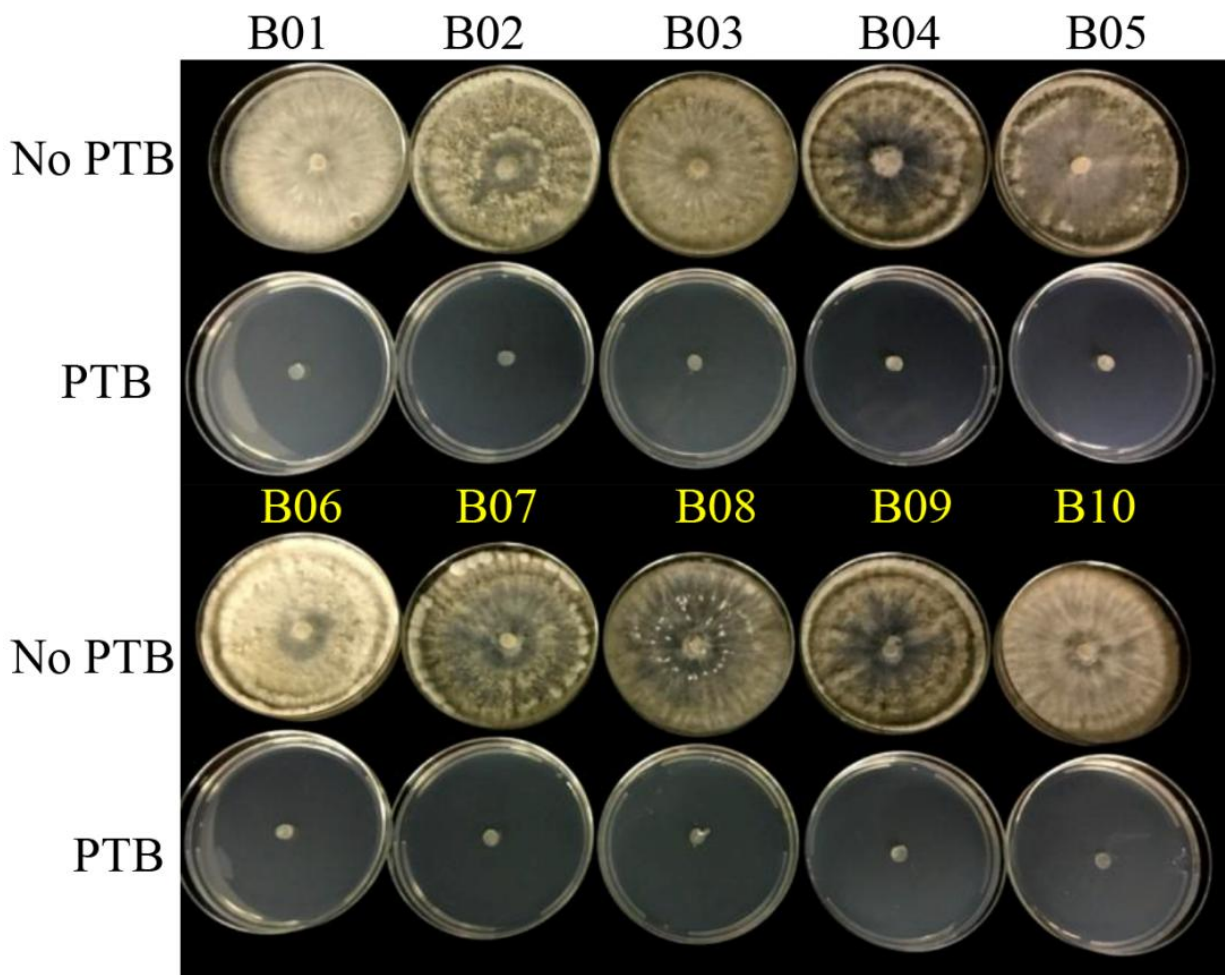


Figure 5.2. Inhibition of mycelial growth of ten *Botrytis* isolates grown on PDA medium containing 25 mM of potassium tetraborate (PTB).

Phytotoxicity APF and PTB on tomato plants

No phytotoxicity was observed on tomato plants treated with 25 mM PTB. In contrast, discoloration of foliage was observed on plants treated with 50 and 75 mM PTB (Figure 5.3). No phytotoxicity was observed on tomato plants treated with APF at 1ml/L (Figure 5.4). These results indicate that PTB and APF can be safely applied at 25 mM and 1ml/L respectively, as preharvest sprays without causing phytotoxicity on greenhouse tomatoes.

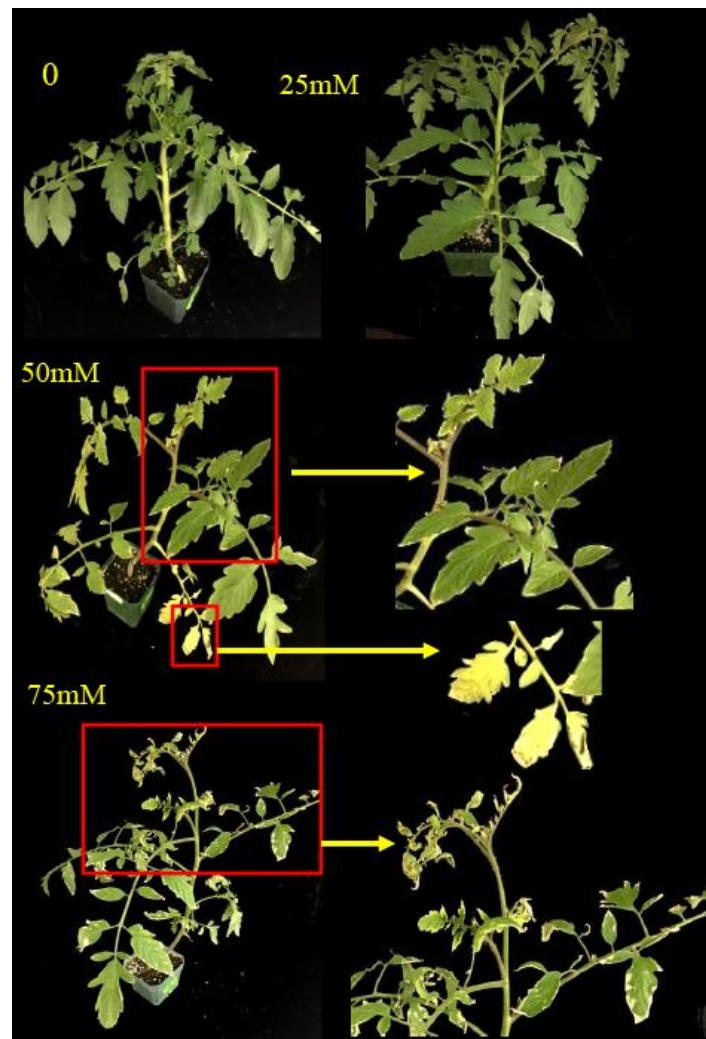


Figure 5.3. Phytotoxicity observed 10 d after spraying 5-week-old- tomato plants with potassium tetraborate (PTB). The figure shows the effect of increased concentrations of potassium tetraborate PTB (25, 50, and 75 mM). Magnified inserts (yellow arrows) show leaves with yellowing, distortion and necrotic margins.



Figure 5.4. Phytotoxicity observed 10 d after spraying 5-week-old- tomato plants with Agrichem Proprietary Formulation APF. The leaves show the effect of increased concentrations of APF (1, 2, and 4 ml/L). Red circles show chlorotic leaves. No phytotoxicity was observed at applications of 1 ml/L.

Effects PTB and APF on gray mold

Both PTB and APF reduced ($P < 0.05$) the severity of gray mold disease on tomato fruit at the turning stage followed by storage at 25 or 4 °C. The severity of gray mold also was reduced by 80% when plants treated with APF at the light red ripening stage at either storage temperature. In contrast, neither PTB nor APF reduced disease severity when tomato plants were treated at the light red ripening stage followed by storage at either temperature (Figures 5.5 & 5.6). Lesions were not observed on fruit treated with PTB or APF at the turning stage of ripening followed by storage at 25 or 4°C (Figure 5.7). The number of marketable fruit increased when plants were treated with APF at the turning and pink ripening stage and stored at 4 or 25°C. Marketability increased following pre-harvest treatment with PTB when sprayed at the turning stage and stored at 4 or 25°C but marketability was not improved when fruit were treated at the pink stage of ripening followed by storage at 25°C (Figure 5.7).

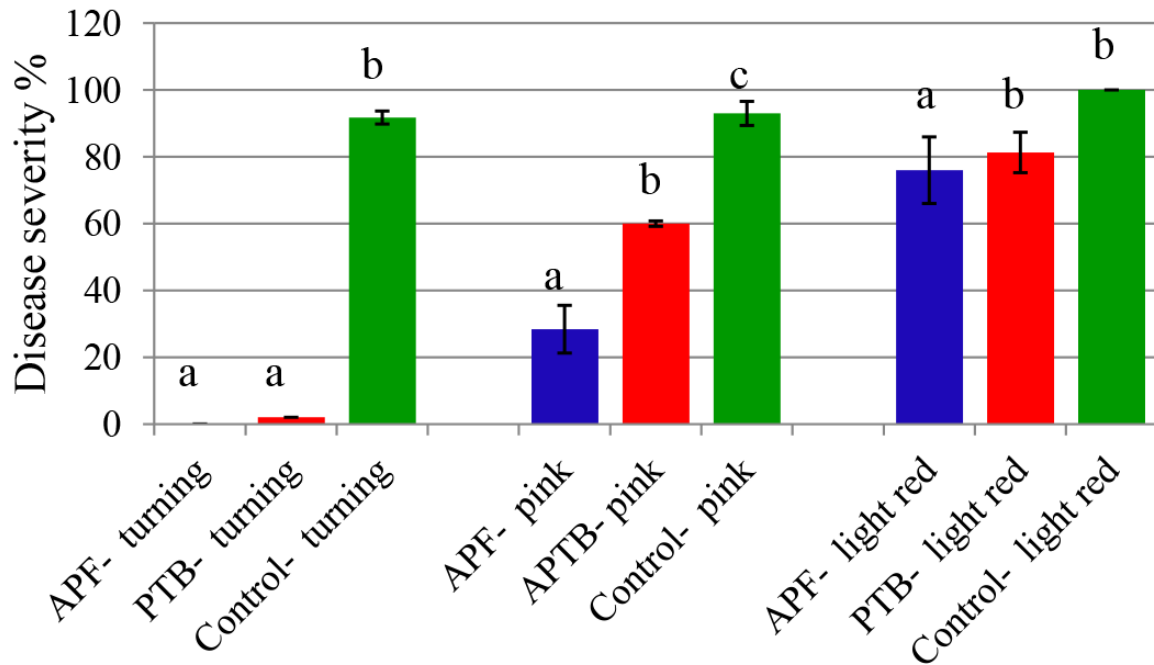


Figure 5.5. Disease severity of gray mold on tomato fruit at postharvest following preharvest spray with either potassium tetraborate (PTB, 25 mM) or a proprietary formulation (APF, 1ml/L) at three ripening stages in the greenhouse. Harvested fruit were stored for 10 days at 25°C. Vertical bars show mean values and standard error (\pm SE). Means with different letters are statistically different according to Duncan's multiple range test ($p < 0.05$).

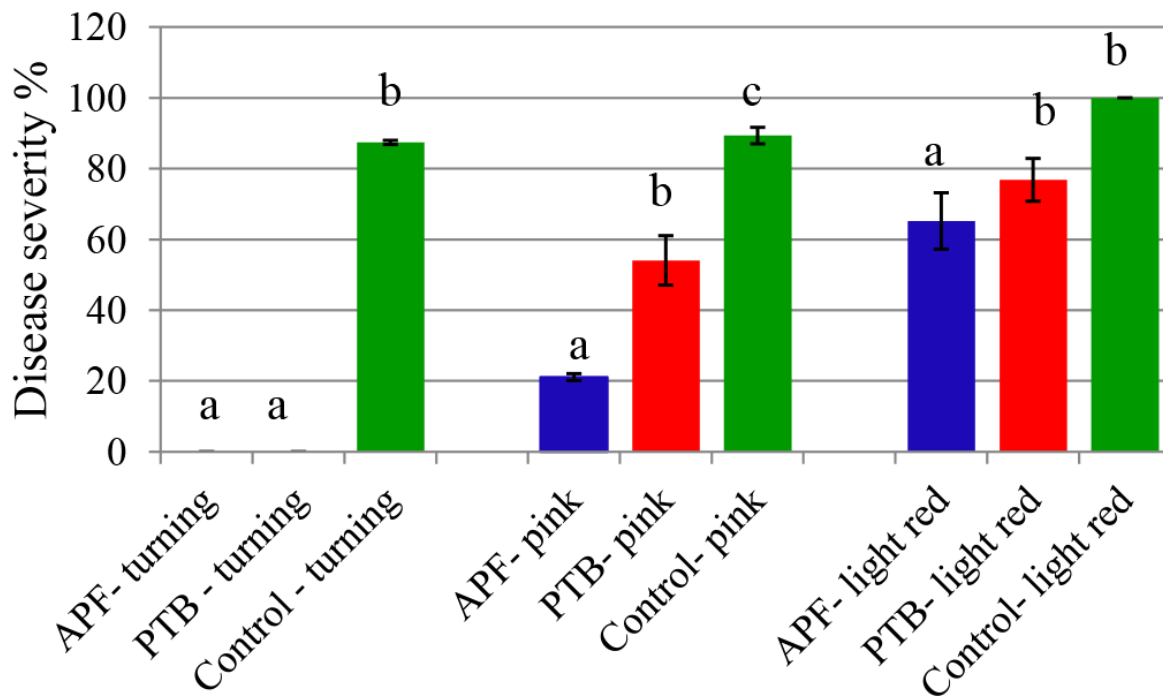


Figure 5.6. Postharvest disease severity of gray mold on tomato fruit following preharvest spray with either potassium tetraborate (PTB, 25 mM) or a proprietary formulation (APF, 1ml/L) at three ripening stages in the greenhouse. Harvested fruit were stored 10 days at 4°C. Vertical bars show mean values and standard error (\pm SE). Means with different letters are statistically different according to Duncan's multiple range test ($p < 0.05$).

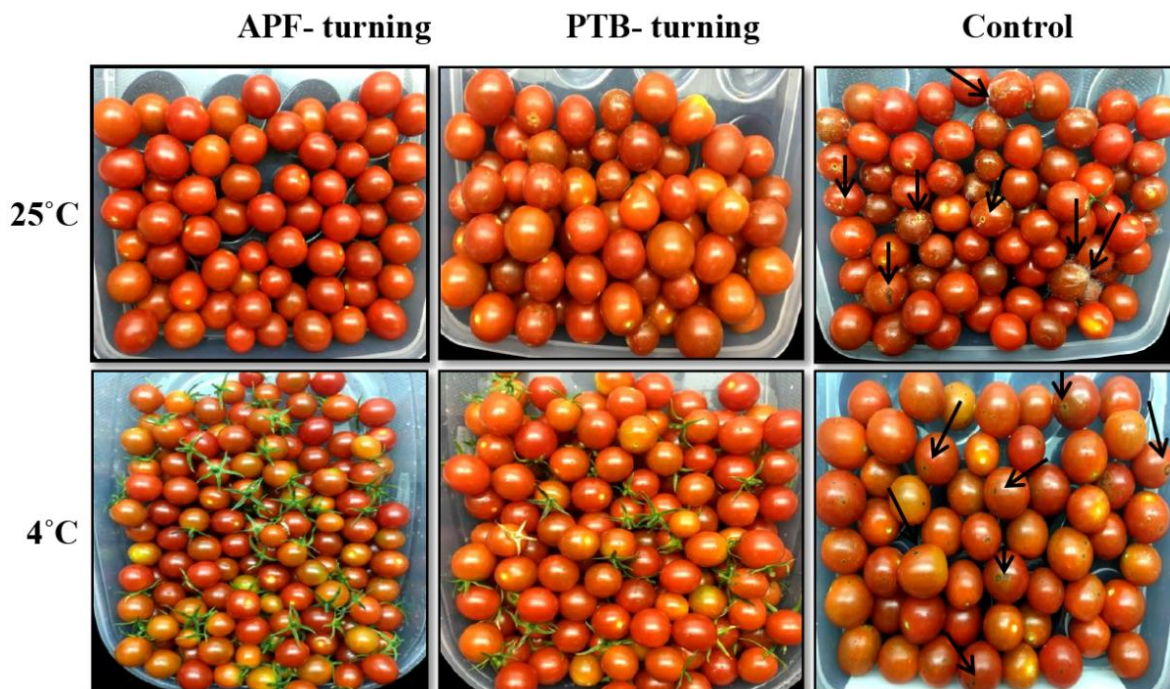


Figure 5.7. Efficacy of a proprietary formula (APF) and potassium tetraborate (PTB) in reducing gray mold disease on tomato fruit when treated at the “turning” stage of ripening. Fruit were stored for 10 days at 4 or 25°C. The photograph was taken 10 days after storage. Arrows point to lesions caused by *Botrytis cinerea*.

Discussion

Boron is one of the essential microelements for plants and improves fruit quality (Plich and Wójcik, 2001). Recently, potassium tetraborate PTB has been used as antifungal compound to control various postharvest diseases including anthracnose caused by *Colletotrichum gloeosporioides* on table grape (Shi et al., 2012) mango (Shi et al., 2011), and gray mold caused by *Botrytis cinerea* on table grape (Qin et al., 2010). In this study, PTB at 25 mM completely inhibited the mycelial growth of all ten *Botrytis* isolates *in vitro*, and this concentration did not show phytotoxicity symptoms on tomato plant leaves. A preharvest application of PTB at the turning stage of ripening tomato in the greenhouse reduced gray mold of postharvest tomato. Corroborated by laboratory results this suggests that disease control in the greenhouse resulted

from the direct antifungal effect of PTB on *Botrytis cinerea*. The results are consistent with another study (Shi et al., 2011) that showed that applications of PTB on mango trees reduced the postharvest incidence of anthracnose. In addition, our previous work showed that immersion of infected tomato fruit in PTB at 100 mM for 10 min reduced the soft rot disease caused by *Pectobacterium carotovorum* (Ahmed et al., 2017b).

Several possible mechanisms to explain the antifungal activity of borate on *Botrytis* include accumulation of reactive species ROS in fungal spores, mitochondrial degradation and cell death, cytoplasmic disintegration, delayed nuclear division, DNA damage, and changing the expression of antioxidant proteins and hydrolytic enzymes (Hipkiss, 2006; Qin et al., 2007; Qin et al., 2010; Shi et al., 2011; Shi et al., 2012). In addition, (Qin et al., 2010) demonstrated that borate showed adverse effects on fungal conidia by decreasing the endocytosis process essential for mycelial tip-growth.

Conclusion

PTB and APF applied at 25 mM or 1ml/L, respectively, were effective against *B. cinerea* in *vitro* assays. Both PTB and APF reduced postharvest gray mold disease incidence when applied in the greenhouse as preharvest sprays to tomato fruits at the turning and pink ripening stages. PTB and APF should be effective in protecting tomato fruit from gray mold that usually develops after harvest. Preharvest application of these products improved fruit quality. Potassium tetraborate has potential use as a safe and cost-effective treatment for disease management of gray mold in the greenhouse tomatoes.

CHAPTER VI

DEVELOPMENT OF GENOME INFORMED DIAGNOSTICS FOR SPECIFIC AND RAPID DETECTION OF PECTOBACTERIUM SPECIES USING RECOMBINASE POLYMERASE AMPLIFICATION COUPLED WITH A LATERAL FLOW DEVICE

Introduction

The soft rot bacterium *Pectobacterium* (formerly known as *Erwinia*) of the family Enterobacteriaceae causes significant economic losses on a wide range of host plants in the field and during storage (Toth et al., 2003). *Pectobacterium* is a representative genus comprising different species that can cause wilt, stem rot, soft rot and postharvest diseases on many fruits and vegetables. Several devastating species of *Pectobacterium* include *P. carotovorum*, *P. atrosepticum*, *P. betavascularum*, and *P. wasabiae* that affect a broad host range of both dicot and monocot plant families (Gardan et al., 2003; Charkowski, 2006). *Pectobacterium* and *Dickeya* of the family Enterobacteriaceae are very similar and share a common host range including potato, tomato, pepper, tobacco, broccoli (Ma et al., 2007; Brady et al., 2012). Kim et al (2009) demonstrated that these pathogens could be found as mixed populations in an infected plant tissue. Discrimination and detection of these plant pathogens is challenging and cannot be identified based on disease symptoms. Methods for identification are essential to determine the sources of contamination. Rapid diagnostic tools that can differentiate *Pectobacterium* from *Dickeya* and other closely related genera in the Enterobacteriaceae are needed.

Traditional bacteriological practices are time consuming, labor intensive, and require trained personal to consistently distinguish genera. Polymerase chain reaction (PCR) has become the most widely used method for accurate detection of plant pathogens (Zanoli and Spoto, 2012).

However, PCR-based methods have some disadvantages. These methods are time consuming, require a sophisticated and expensive thermocycler, and cannot be used at point-of-care (Wang et al., 2009). Recent advancements in isothermal methods such as loop-mediated isothermal amplification (LAMP), are rapid, sensitive, accurate and do not require a thermocycler. Yasuhara-Bell et al. (2016) reported the development of a specific loop-mediated isothermal amplification (LAMP) assay to detect the blackleg pathogen, *P. atrosepticum*, and soft rot pathogen, *P. carotovorum*. However, the LAMP method also has disadvantages including complexity in primer design, a high temperature requirement of 65°C, and a relatively expensive portable device for test runs. Recombinase polymerase amplification (RPA) has gained popularity in recent years. This is a sensitive and accurate isothermal detection method and can be performed at the point-of-care without the need for an expensive instrument. RPA has an added advantage in that it is unaffected by plant inhibitors (M. Arif, *personal communication*).

The objective was to develop a RPA assay for specific and rapid detection of *Pectobacterium*. The assay includes host tissue (tomato and potato) as an internal control to enhance reliability and accuracy.

Materials and methods

Source of bacterial strains and DNA isolation

The bacterial strains used in both inclusivity and exclusivity panels are listed in Table 6.1. Strains were stored in -80°C and re-grown on TZC medium (10 g/L peptone, 5 g/L glucose 17 g/L agar, and 0.001% 2, 3, 5-triphenyle-tetrazolium chloride added after autoclaving). Plates were incubated at 26°C±2°C. The Wizard Genomic DNA Purification Kit (Promega, Madison, WI) was used to extract the genomic DNA from all strains according to the manufacturer's protocols. The concentration of extracted DNA was measured using a NanoDropTM 2000C

(Thermo, Fisher Scientific Inc, Worcester, MA). Extracted DNA was stored in a freezer at -20°C. For host samples, healthy peppers, eggplants, tomatoes, and potato were grown in the greenhouse. Fruit and tuber were surface sterilized with 10% sodium hypochlorite for 1 min and washed three times with sterilized water. A plant tissue kit (Promega) was used to extract the genomic DNA from all fruit samples following the manufacturer's protocols.

Table 6.1. Bacterial strains used in this study.

Lab ID	Original ID	Organism	Location	Host	R P A
A5280	1-#31	<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>	Hawaii	Irrigation water	+
A5278	1-#21	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	Hawaii	Irrigation water	+
A5368	5X	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	Hawaii	<i>Aglaonema</i> sp.	+
A5366	3C	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	Hawaii	<i>Aglaonema</i> sp.	+
A5371	CC26	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	Hawaii	<i>Aglaonema</i> sp.	+
A3048	E60	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	Hawaii	Cabbage	+
5349	5C	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	Hawaii	<i>Aglaonema</i> sp.	+
5352	T-15	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	Hawaii	<i>Aglaonema</i> sp.	+
A5347	2-2b	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	Hawaii	<i>Aglaonema</i> sp.	+
A6273	BA17	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	Hawaii	Tomato fruit	+
A5354	11X	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	Hawaii	<i>Aglaonema</i> sp	+
A6149	WPP5	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	Wisconsin	Potato	+
A6197	ER1	<i>P. carotovorum</i>	Hawaii	Cabbage	+
A1089	EC153	<i>P. carotovorum</i> subsp. <i>odoriferum</i>	California	Pepper	+
A1847	IPM 60	<i>P. carotovorum</i> subsp. <i>odoriferum</i> ^a	Colorado	Potato	+
A2686	E43	<i>P. carotovorum</i> subsp. <i>odoriferum</i>	New Zealand	Aroid	+
A5359	EC	<i>P. carotovorum</i> subsp. <i>odoriferum</i>	Hawaii	Papaya	+
A6156	WPP16	<i>P. carotovorum</i> subsp. <i>odoriferum</i> ^b	Wisconsin	Potato	+
A6154	WPP12	<i>P. carotovorum</i> subsp. <i>odoriferum</i> ^b	Wisconsin	Potato	+
A2688	E45	<i>P. carotovorum</i> subsp. <i>brasiliensis</i>	New Zealand	Aroid	+
A6151	WPP20	<i>P. carotovorum</i> subsp. <i>brasiliensis</i>	Wisconsin	Potato	+
A6152	WPP165	<i>P. carotovorum</i> subsp. <i>brasiliensis</i>	Wisconsin	Potato	+
A1850	IPM 260	<i>P. atrosepticum</i> ^c	Colorado	Potato	+
A1852	M784	<i>P. wasabiae</i> ^c	Colorado	Potato	+
A6159	WPP168	<i>P. wasabiae</i>	Wisconsin	Potato	+
A6166	Ecb2	<i>P. wasabiae</i> ^d	California	Beta vulgaris	+
A6056	3-L	<i>Dickeya zeae</i>	Hawaii	Pineapple	-
A5422	CFBP2052	<i>D. zeae</i>	USA	<i>Zea mays</i>	-
A5423	CFBP6466	<i>D. zeae</i>	Malaysia	Pineapple	-
A5642	CFBP 3855	<i>D. dadantii</i>	France	<i>Saintpaulia</i>	-
A5641	CFBP 1270	<i>D. chrysanthemi</i>	Denmark	<i>Parthenium</i>	-
A5415	CFBP2048	<i>D. chrysanthemi</i>	USA	<i>Chrysanthemum</i>	-
A5582	PRI 2188	<i>D. solani</i> ^f	Israel	Potato	-
A5581	PRI 2187	<i>D. solani</i> ^f	Israel	Potato	-
A2058	H-160	<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	Idaho	Tomato	-
A6095	20037	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Nebraska	Corn	-
A3617	CC93R	<i>Xanthomonas vesicatoria</i>	South America	Tomato	-
A5491	EB2	<i>Ralstonia solanacearum</i>	Indonesia	Tomato/Eggplant	-

a. Originally (1987) identified as *Erwinia carotovora* subsp. *carotovora*

b. Originally (2001) identified as *Pectobacterium carotovorum* subsp. *carotovorum*

c. Originally (1987) identified as *Erwinia carotovora* subsp. *atroseptica*

d. Originally (2001) identified as *Pectobacterium betavascularum*

e. Originally (2004) identified as *Erwinia chrysanthemi*

f. Originally (2007) identified as *Erwinia* sp.

Endpoint PCR and *dnaA* sequencing

The *dnaA* region of bacterial strains was amplified using primer sets designed for *Clavibacter*, *Dickeya*, *Pectobacterium*, *Ralstonia*, and *Xanthomonas* (Table 6.2) (Schneider et al., 2011). A 20 µl of PCR reaction contained 10 µl of GoTaq Green Master Mix (Promega), 0.5 µl of 10 mM of each forward and reverse primer, 1 µl of template DNA, and 8 µl of Ultra Pure DNase/RNase-Free distilled water (Thermo Fisher Scientific). PCR was performed with initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of 94°C for 20 seconds, 60°C for 20 seconds and 72°C for 20 seconds, followed by a three minutes extension at 72°C and hold at 12°C. Agarose gel (1.5 %) electrophoresis was used to separate all PCR amplicons, stained with 0.4 µg/ml ethidium bromide, and bands visualized with a UV illuminator. PCR was performed in a T100 Thermal cycler (Bio-Rad, Hercules, California). Amplified PCR products were treated using ExoSAP-1T (Affymetrix, Inc., Santa Clara, California). A 5 µl of post-PCR reaction and 2 µl ExoSAP-IT reagents were combined and incubated at 37 °C for 15 min following by an incubation of 80 °C for 15 min. Each treated template sequence both sense- and antisense-strands using genus specific primers. Sequencing was performed at the Advanced Studies in Genomics, Proteomics and Bioinformatics facility (ASGPB), Honolulu, HI.

Sequence analyses

The sequences of partial *dnaA* gene regions of tested strains were manually edited in order to generate error free consensus sequences for all the strains. Consensus sequences generated after manual editing were compared against the NCBI GenBank nucleotide database using the BLASTn algorithm. Consensus sequences were aligned and used to reveal the phylogenetic relationships among the strains. Geneious 17.1.7 was used for editing, alignment,

and generation of phylogenetic trees. The Sequence Demarcation Tool was used to calculate percent similarity among the strains.

Genus-specific *Pectobacterium* primer and probe design

The genomes of *P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *odoriferum*, *P. atrosepticum*, *P. wasabiae*, *Dickeya zaeae*, *D. dadantii*, *D. solani*, *Erwinia amylovora*, *Clavibacter michiganensis* subsp. *michiganensis*, *C. michiganensis* subsp. *nebraskensis*, *Xanthomonas vesicatoria* and *Ralstonia solanacearum* were retrieved from NCBI GenBank genome database. Genomes within *Pectobacterium* and *Dickeya* were aligned with progressive Mauve alignment using Mauve (2.4.0). NC_012917 was used as a reference genome. Generated locally Collinear Blocks (LCBs) were analyzed to search for unique regions for *Pectobacterium* spp. Regions within the *tyrR* family transcriptional regulator gene were selected to design RPA primers and probe to specifically detect all *Pectobacterium* spp. RPA primers and probes were designed manually and checked for thermodynamic characteristics following the

Table 6.2. Primers used in this study (Schneider et al., 2011).

Primer name	Forward primer	Reverse primer
Clav.dnaA	5-TACGGCTTCGACACCTTCG-3	5-CGGTGATCTTCTTGGTGGCG-3
Dic. dnaA	5-CACACYTATCGYTCCAAYGT-3	5-TGTCGTGACTTTCYTCRCGC-3
Pec. dnaA	5-ATGTGAACCCSAAACATACGT-3	5-TTCACGCAACTGCTCAATCTT-3
Ral.dnaA	5-TCRCGSCTGAACCSATCCT-3	5-TTGAGCTGSGCGTCCTTGC-3
Xan.dnaA	5-CAGCACGGTGGTGTGGTC-3	5-CCTGGATTTCGATTACACC-3

parameters described by (Arif and Ochoa-Corona, 2013). The primer and probe locations were represented using the BLAST Ring Image Generator (BRIGS) (Alikhan et al., 2011). Average Nucleotide Identity (ANI) was calculated using Orthologous Average Nucleotide Identity Tool (OAT). The reverse primer PCRT-RPAR1 was labeled with biotin at 5' position and the probe was labeled with FAM. Primers and probes for RPA (target pathogen and host) are listed in Table 6.3. Primers were synthesized by Integrated DNA Technologies Inc. (IDT, Coralville, IA) and the probes were from Biosearch Technologies Inc., Novato, CA (Table 6.3). The Internal Transcribed Spacer (ITS) region was used to design the primers and probe to target host genome of tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*). The primers and probes details are given in Table 6.3.

RPA assay

A 50 µl reaction was performed with the TwistAmp Exo® kit following the manufacturer's protocols. Each reaction included 29.5 µl of rehydration buffer, 0.6 µl of (10 µM) of Twist Amp LP probe, 2.1 µl (10 µM) of forward primer, 2.1 µl (10 µM) of biotin-labeled reverse primer, 11.2 µl nuclease-free water, 2 µl of DNA template, 2.5 µl of magnesium acetate. RPA reactions were performed at constant temperature of 37°C using for 30 minutes and the PCR machine used for 39°C incubation. Each reaction was conducted with a non-template control (NTC). After amplification, 2 µl of RPA product was added to a mix of 400 µl of nuclease-free water and 100 µl of buffer (Milenia Biotec, Germany). A Lateral Flow Device (LFD) was vertically inserted into the dilution mix and left for 5 min.

Table 6.3. List of primers and probes for the RPA lateral-flow strip developed for this study.

Primer name	Sequence 5'–3'	Length	GC content%
PCRT-RPAF1	CTGGATATGAAAGGGAAACCGGAGT TATTTAATCC	35	40
PCRT-RPAR1	Biotin- GTCATTTCCAGCAAGAAATCCTGACC GCGAATCA	35	46
PCRT-LP	FAM- TGTTTGAGCAATCGGCAGAGACCATC AGCGATTTGACGATTGGCAGCA	48	50
IC-RPAF1	AACACAAACGACTCTCGGCAACGGA TATCTCG	32	50
IC-RPAR1	Biotin- ATGGCTTCGGGCGCAACTTGCGTTCA AAGACT	32	53
IC-LP	TGAAGAACGTAGCGAAATGCGATAC TTGGTHTGAATTGCAGAATCCCGTGA	50	44

Bacterial-infected fruit materials and DNA extraction (internal control)

Individual tomato fruit were individually inoculated in a biosafety hood with five bacterial strains of *Pectobacterium* sp. A5280, A5278, A5368, A2688, and A3048 using 10 µl of 10⁸ (CFU/ml) of each bacterial suspension for each stab-wound inoculation. Individual potato tubers were inoculated with the *Pectobacterium* strains A5278, A5368 and *Dickeya* sp. strains A5415, A5581, and A5582. Fruits and tubers (ten in total) were incubated for 24 h at 28°C and DNA was extracted using the Wizard Genomic DNA Purification Kit following the

manufacturer's protocols. Each of the 10 infected samples was assayed with each of the pathogen- and host-targeted primers and probes.

RPA sensitivity and spiked assays

DNA of *P. carotovorum* subsp. *carotovorum* strain A5280 was serially diluted 10-fold to prepare 1 ng to 1 fg per μ l dilutions as determined using a Nano Drop. Likewise, the plant host genomic DNA was prepared at a concentration of 98 ng/ μ l. The detection limits of the RPA assay were evaluated on samples containing the pathogen DNA alone or samples containing the pathogen DNA mixed with 1 μ l of host DNA for each reaction.

Results

Target selection *in-silico* validation

Whole genome sequences of *Pectobacterium* and other genera downloaded from NCBI GenBank genome database were explored for unique and conserved target selection for *Pectobacterium*. The *P. carotovorum* genome accession number NC_012798 was used as a reference for whole genome alignment and analysis. TyrR family transcription regulator gene (TyrR) was selected and targeted for RPA primers and probes design. Designed primers and probes were blasted against the NCBI GenBank database for *in-silico* validation. There were no similar sequences existed with other genera including *Dickeya*. The primer and probe location in the genome was completed and visualized in a ring image (Figure 6.1). The ring image output showed a comparison of a reference genome sequence of *P. carotovorum* colored with dark blue in the center (inner most) circle with other closely and distantly related bacterial genomes *P. carotovorum* subsp. *carotovorum* (18525), *P. carotovorum* subsp. *brasiliensis* (20350), *P. atrosepticum* (9125), *P. wasabiae* (13421), *Dickeya zeae* (6929), *D. dadantii* (14500), *D. solani*

(9460), *Erwinia amylovora* (13961), *Xanthomonas vesicatoria* (18470), *Ralstonia solanacearum* (32295), *Clavibacter michiganensis* subsp. *michiganensis* (9480) were included in this analysis from the inner-most into the outermost circle (Figure 6.1). The orthologous average nucleotide tool predicted overall similarity among all the tested bacterial genera. Within the species of *Pectobacterium*, 88.9 – 90.8% similarity was calculated (Figure 6.2) whereas the closely related genus, *Dickeya*, showed 74.9-76.2% similarity with *Pectobacterium* species. A low nucleotide similarity was observed with unrelated species, *E. amylovora* X. *vesicatoria* and *Clavibacter michiganensis* subsp. *michiganensis* (Figure 6.2).

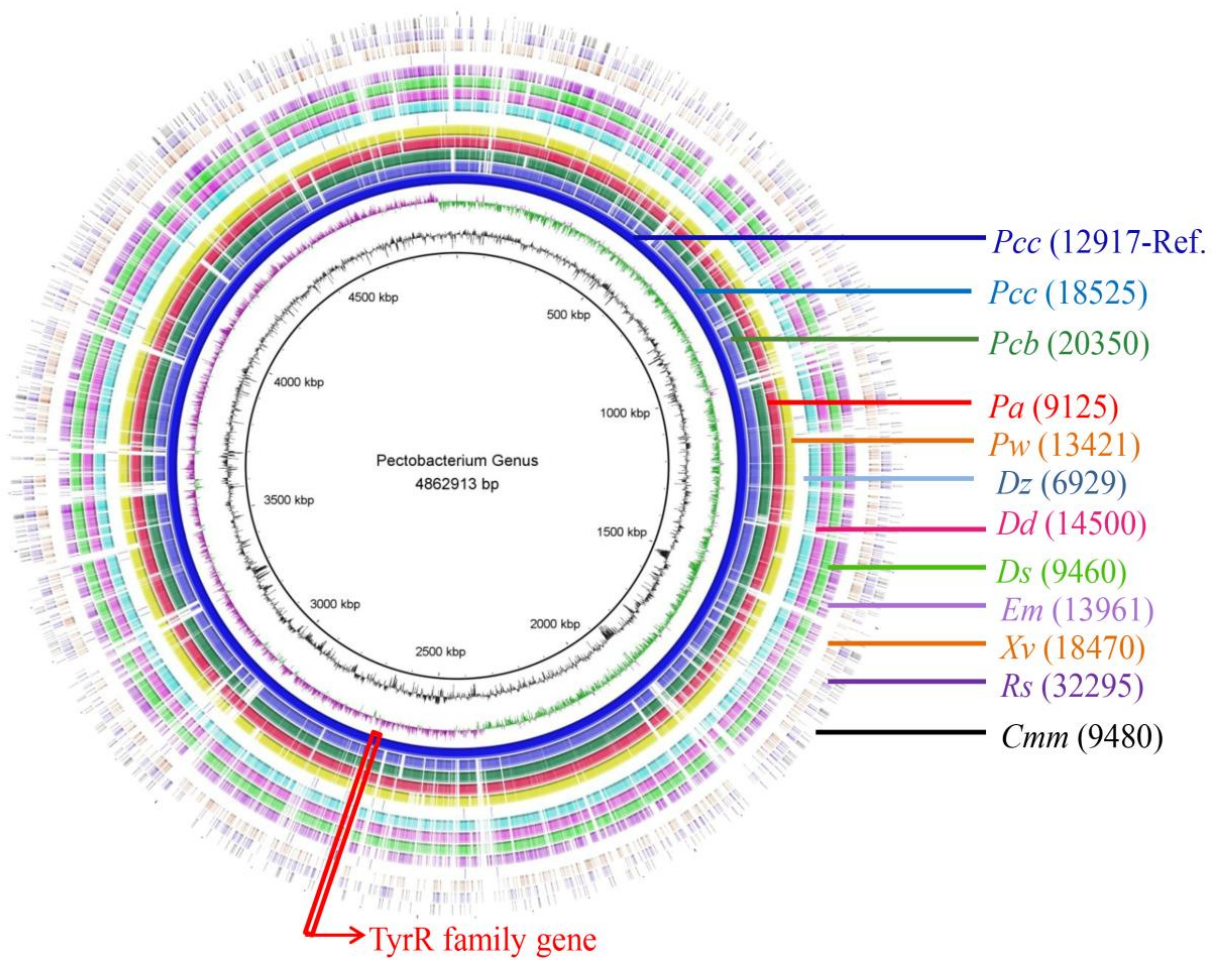


Figure 6.1. Ring image of all bacterial genomes used in this study showing the location of tyrR gene which was used to design recombinase polymerase amplification (RPA) primers and probe. ***Pcc-Ref.*** (*Pectobacterium carotovorum* subsp. *carotovorum*); ***Pcc*** (*Pectobacterium carotovorum* subsp. *carotovorum*); ***Pcb*** (*Pectobacterium carotovorum* subsp. *brasiliensis*; ***Pa*** (*P. atrosepticum*); ***Pw*** (*P. wasabiae*); ***Dz*** (*Dickeya zeae*); ***Dd*** (*D. dadantii*); ***Ds*** (*D. solani*); ***Ea*** (*Erwinia amylovora*); ***Xv*** (*Xanthomonas vesicatoria*); ***Rs*** (*Ralstonia solanacearum*); ***Cmm*** (*Clavibacter michiganensis* subsp. *michiganensis*) were included in this analysis.

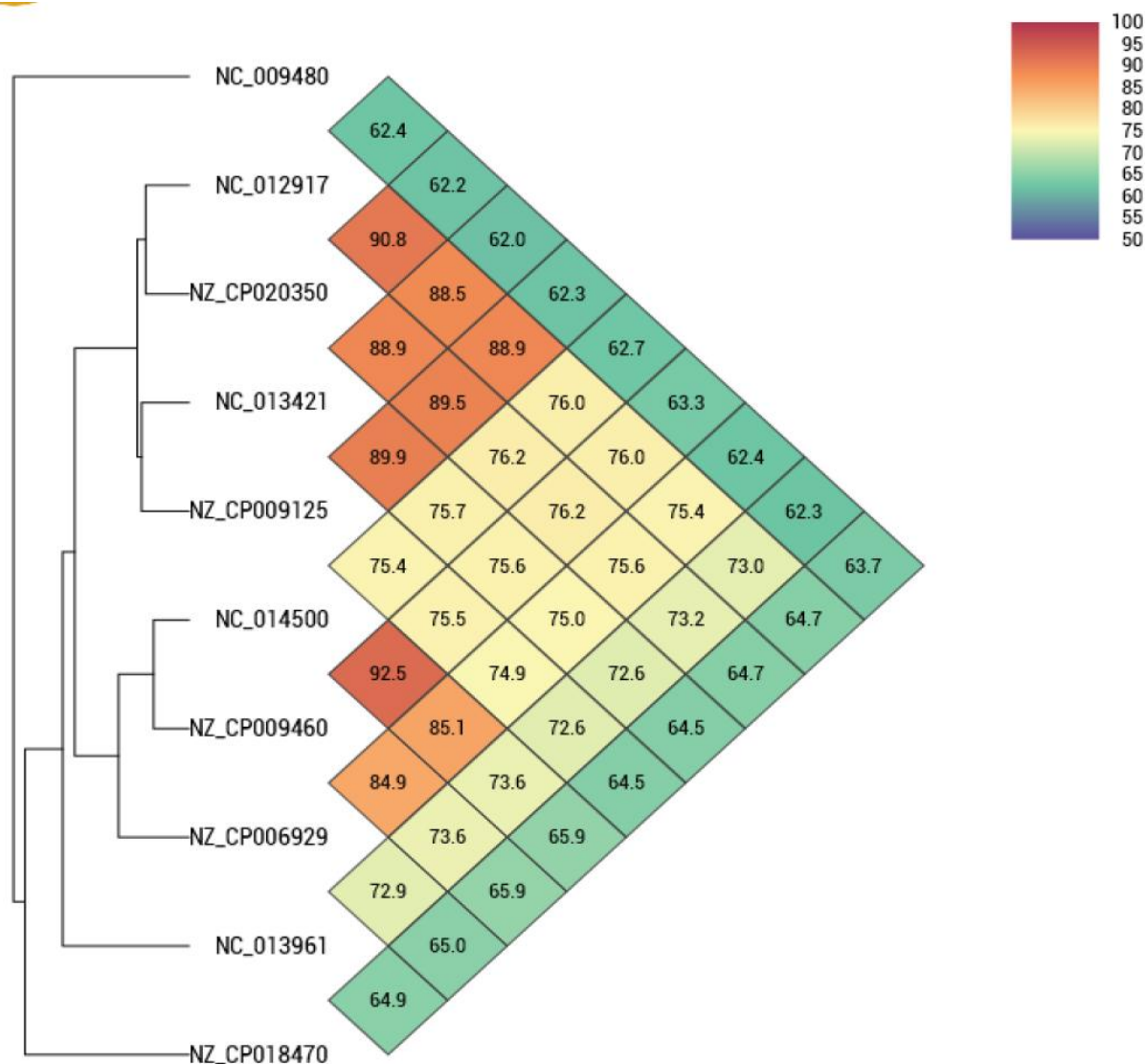


Figure 6.2. Orthologous average nucleotide identity tool used to measure overall similarity among all bacterial genera used in this study. Values in different colors boxes indicate the similarity percentage among genomes. *Clavibacter michiganensis* subsp. *michiganensis* (009480); *Pectobacterium carotovorum* subsp. *carotovorum* (012917); *P. carotovorum* subsp. *brasiliensis* (CP020350); *P. wasabiae* (013421); *P. atrosepticum* (CP009125); *Dickeya dadantii* (014500); *D. solani* (CP009460); *D. zeae* (CP006929); *Erwinia amylovora* (013961); *Xanthomonas vesicatoria* (CP018470).

Specific target of *Pectobacterium* sp.

The broad range detection capabilities of the RPA assay for *Pectobacterium* was assessed with 30 different strains of three *Pectobacterium* species (*P. carotovorum*, *P. atrosepticum*, and *P. wasabiae*) whereas, the exclusivity panel included strains from different species (*Clavibacter michiganensis* subsp. *michiganensis*, *Dickeya zeae*, *D. dadantii*, *D. solani*, *Ralstonia solanacearum*, and *Xanthomonas vesicatoria*). No false negative or false positives were observed with the lateral flow device. Primers and probes specifically detected only *Pectobacterium* strains (Figure 6.3), indicating that the developed RPA assay was accurate, robust and specific for detection of *Pectobacterium*.

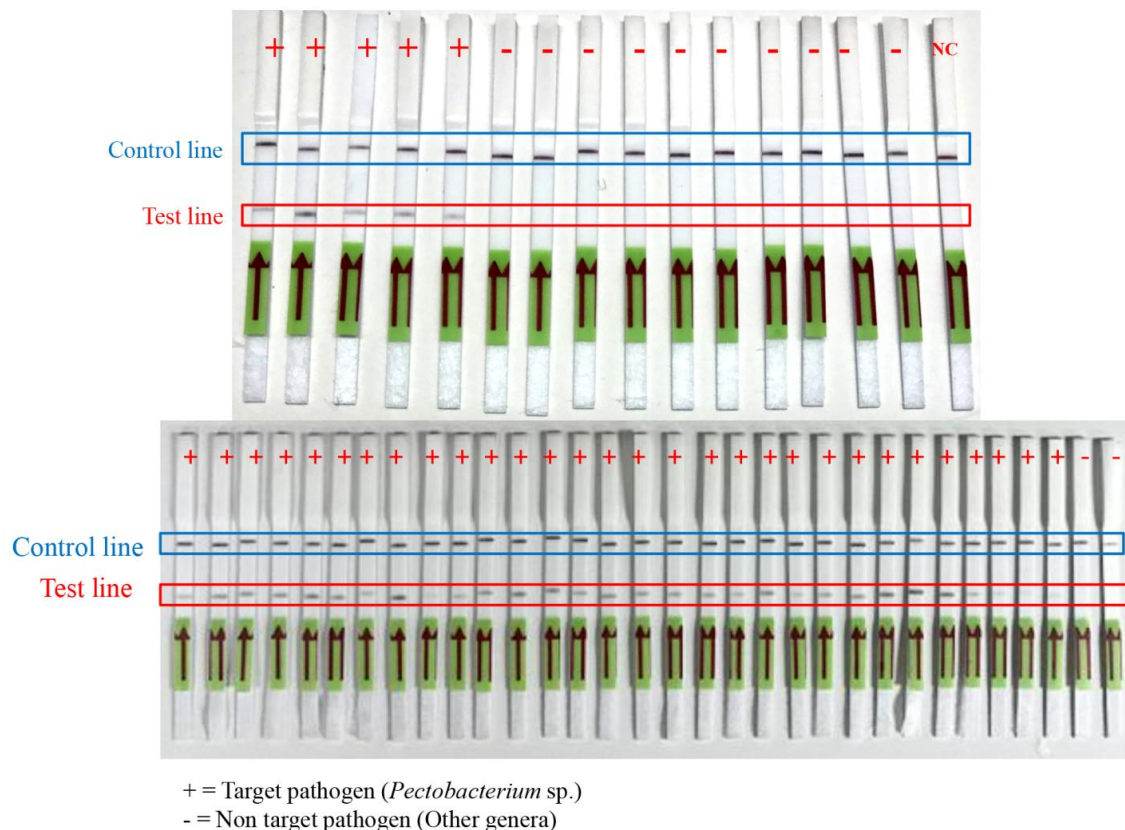


Figure 6.3. The analytical specificity of RPA assay for target pathogen (*Pectobacterium* sp. strains). The test indicated that only the DNAs of *Pectobacterium* sp. reacted positively.

Internal control

RPA primers and probe were designed to enhance the reliability and accuracy of the developed assays. Primers were designed to detect the plant hosts tissue of tomato fruit and leaves, potato tubers, fruit of pepper eggplant as internal controls. Target host tissue produced the positive results and the water (Negative control) showed no reactivity (Figure 6.4). These primers and probes reacted with the tested *Solanum* hosts and were detected on lateral flow strips.

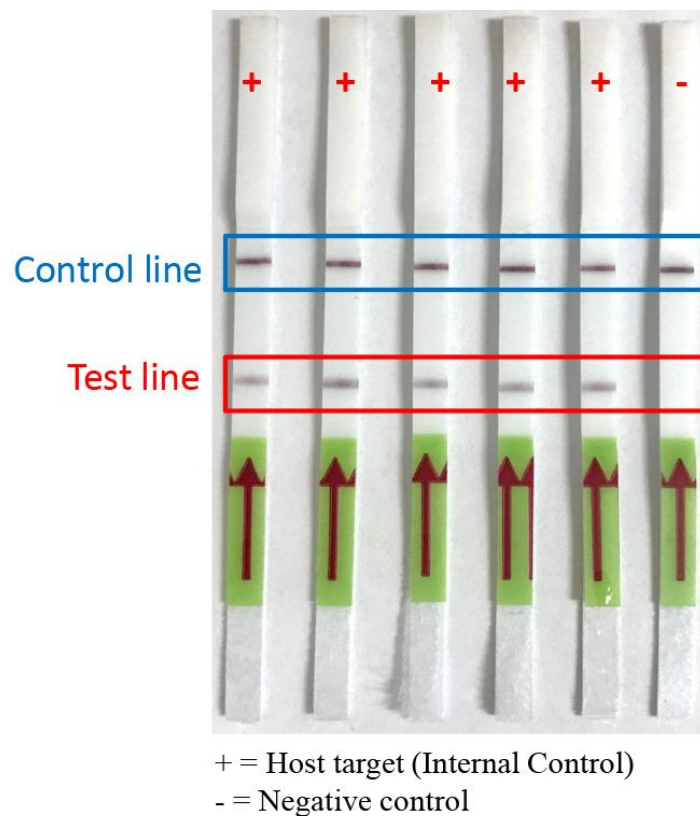


Figure 6.4. The analytical specificity of RPA assay for healthy hosts target (from left to right): tomato leaves; tomato fruit (*Solanum lycopersicum*); potato tuber (*Solanum tuberosum*); pepper fruit (*Capsicum annuum*); eggplant fruit (*Solanum melongena*) and negative (water) control.

Detection of *Pectobacterium* sp. in infected host tissue

The specificity of RPA was evaluated with infected tomato fruit and potato tubers. All tomatoes were inoculated with five different strains of *P. carotovorum* whereas three potato tubers were inoculated with only two of the *P. carotovorum* strains. Three other potatoes were individually inoculated with a strain of *D. chrysanthemi* or *D. solani*. Positive results were obtained only from tissues of tomato and potato infected with *P. carotovorum* whereas no test line was observed with potato tissues infected with either of the *Dickeya* species (Figure 6.5). These results confirmed that the developed RPA assay was specific for *Pectobacterium*. Each infected sample was further tested with host RPA primers and probe as an internal control to confirm the accuracy in DNA isolation. All showed positive results with DNA isolated from infected tissues. No amplification occurred on the negative control (Figure 6.6).

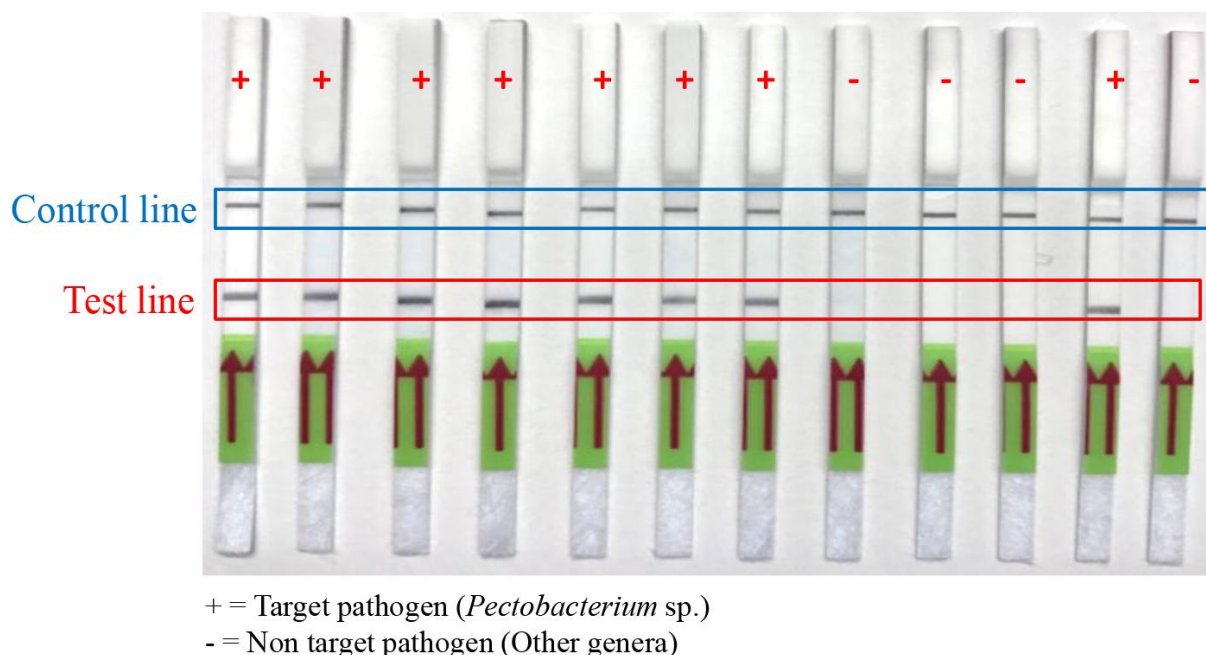
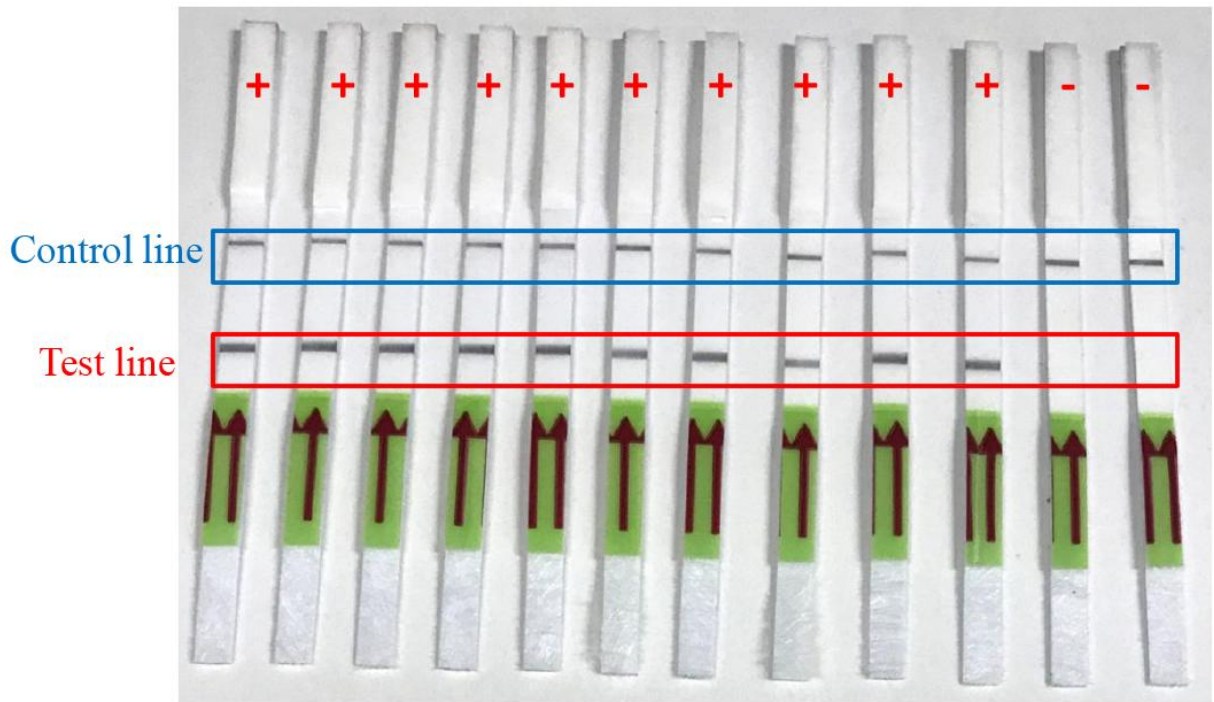


Figure 6.5. The specificity of the RPA assay for *Pectobacterium* using a pathogen-specific primer set in infected plant samples. From left to right: *Pectobacterium carotovorum* subsp. *carotovorum* (A5280); *Pectobacterium carotovorum* subsp. *carotovorum* (A5278); *Pectobacterium carotovorum* subsp. *carotovorum* (A5368); *Pectobacterium carotovorum* subsp. *brasiliensis* (A2688); *Pectobacterium carotovorum* subsp. *carotovorum* (A3048) and potato tubers inoculated with *Pectobacterium carotovorum* subsp. *carotovorum* (A5278); *Pectobacterium carotovorum* subsp. *carotovorum* (A5368); *Dickeya chrysanthemi* (A5415); *Dickeya solani* (A5581); *Dickeya solani* (A5582). Only DNAs of *Pectobacterium* sp. reacted positively and produced a line on strips.



+ = host target (internal control)

- = Non host target

Figure 6.6. The analytical specificity of the RPA assay using the host target primer set for solanaceous hosts. Lines 1 to 5 from (left to right) were *Pectobacterium* strains (A5280, A5278, A5368, A2688, A3048) inoculated into tomato; lines 6 to 10 were potato tubers inoculated with *Pectobacterium* strains (A5278, A5368), and *Dickeya* strains (A5415, A5581, A5582). Line 11 is water only; line 12 is *Pectobacterium* DNA alone. Only DNAs of the hosts (tomato and potato) reacted positively and showing line on strips.

Sensitivity assays

Serially diluted (1 ng to 1 fg) genomic DNA of *P. carotovorum* subsp. *carotovorum* (A5280) was used to perform the sensitivity assay while 1 µl (59 ng/ µl) of healthy tomato host DNA was added in each reaction to complete a spiked sensitivity assay (containing host and bacteria). The assays detected bacterial in all samples to 10 fg of *P. carotovorum* subsp. *carotovorum* DNA (Figure 6.7). The outcome sensitivity detection limit was not affected when 1 µl of tomato host DNA was added to each reaction (Figure 6.8)

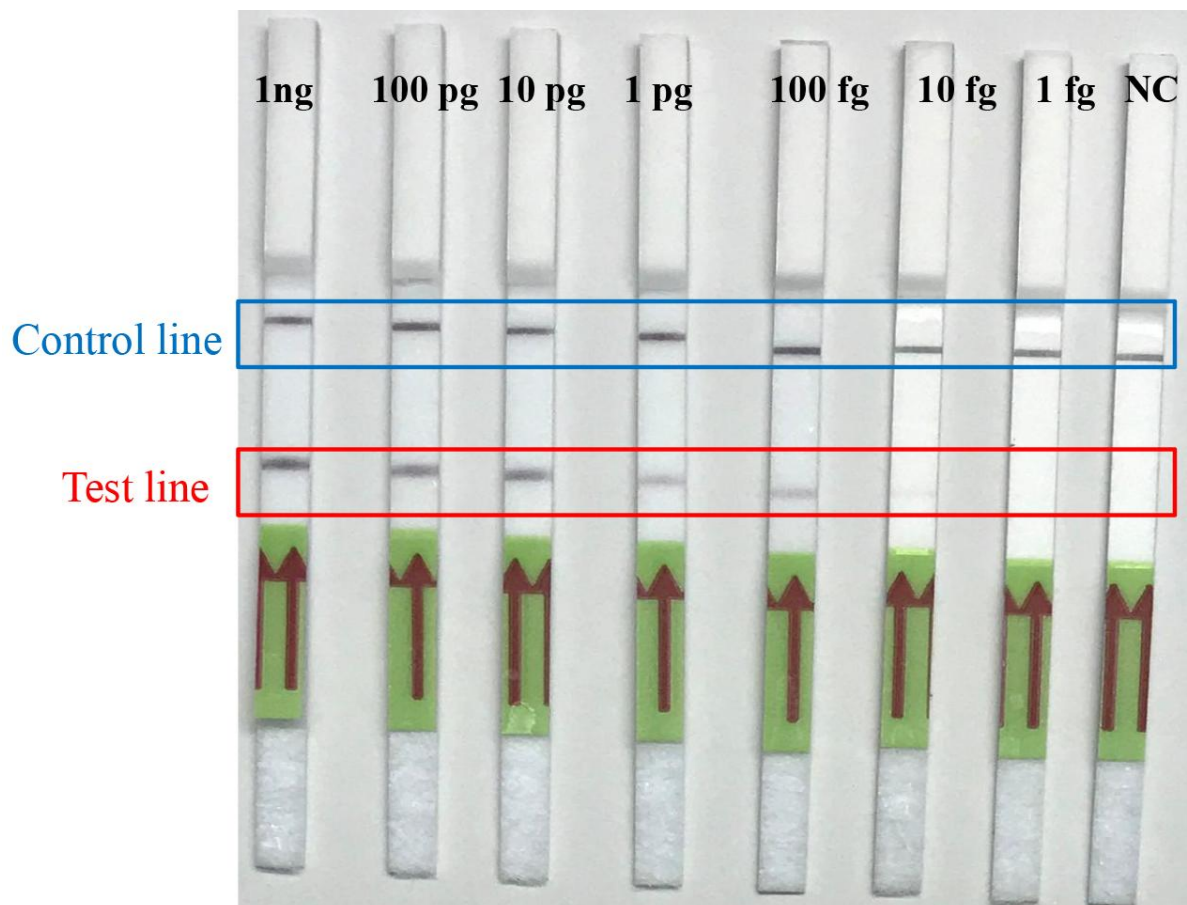


Figure 6.7. Analytical sensitivity of the RPA assay using a serial dilution of *Pectobacterium carotovorum* subsp. *carotovorum* DNA (A5280) ranging from 1 ng to 1 fg.

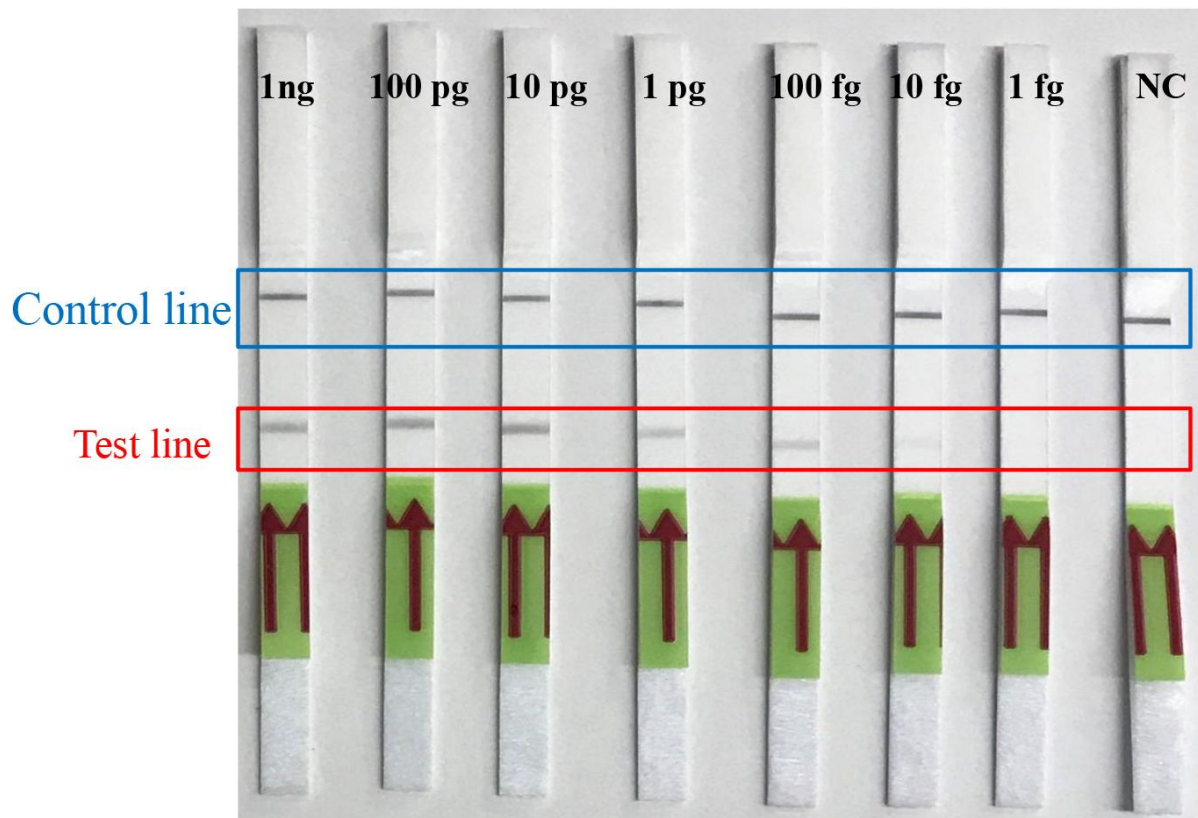


Figure 6.8. Analytical sensitivity of the RPA assay using serial dilution of *Pectobacterium carotovorum* subsp. *carotovorum* DNA (A5280) mixed with tomato host (*Solanum lycopersicum*) DNA from 1 ng to 1 fg.

Discussion

We developed and validated a recombinase polymerase amplification (RPA) assay to detect and discriminate the genus *Pectobacterium* from other closely related genera including *Dickeya* sp. and non related bacterial genera such as *Clavibacter*, *Xanthomonas*, and *Ralstonia*.

LAMP methods for detection of the closely related genera *Pectobacterium* and *Dickeya* have already been developed by Yasuhara-Bell et al. (2016) but LAMP assays have the disadvantage of requiring a high temperature for amplification (65°C) and an expensive instrument. RPA is a comparatively new isothermal technique that can detect target DNA in 15-30 min at 37-42°C with no requirement of sophisticated and/or expensive instruments (Deng and Gao, 2015). In addition, RPA displays greater resistance to inhibitors and can be used with samples of greater complexity as compared to other isothermal methods (Rosser et al., 2015; Moore and Jaykus, 2017). RPA is less affected by inhibitors than the well known LAMP method (M. Arif, unpublished). Twist Dx RPA reagents are available in lyophilized form and along with the strips. The entire kit can be easily transported to the field without the need for cold storage. Results are easily visualized and interpretation does not require specific training or instrumentation. Ouyang et al. (2013) developed a Razor Ex-based sensitive field detection method for the plant pathogenic bacterium, *Xylella fastidiosa* subsp. *pauca* but its use required an expensive Razor Ex instrument and training for operation. Reagent cost per reaction was high for this instrument and limited the use of this method routine in-field diagnostics (Arif et al, 2014).

Due to its high sensitivity, robustness and accuracy the RPA methods using a lateral flow device is an excellent option for both in and off lab detection of *Pectobacterium*. Accurate and

rapid detection and identification of a disease-causing bacteria is one of the most significant prerequisite aspects of disease control (Riley et al., 2002). The successful application of RPA assay for detection of *Pectobacterium* sp. using a primer set and probe targeting TyrR family transcriptional regulator gene was demonstrated in this study. Specificity results with a wide range of isolates in inclusivity panel and isolates from both closely- and distantly-related plant pathogenic bacterial species showed no false positive outcomes. Moreover, no cross reactivity was observed when potato tuber tissues were infected with *Dickeya chrysanthemi*, *D. solani*, and *D. zeae* whereas tomato and potato tissues infected with *Pectobacterium carotovorum*, *P. atrosepticum*, and *P. wasabiae* were all positive, confirming the presence of *Pectobacterium* sp.

Diagnostic methods should be sufficiently sensitive to detect a minimum amount of the target pathogen in an infected sample to eliminate the chances of obtaining false negative results. The developed RPA assay was sensitive, detecting 10 fg of pure *Pectobacterium* DNA and *Pectobacterium* DNA mixed with host DNA. The detection limit was the same for *Pectobacterium* DNA as well as bacterial DNA mixed with host DNA indicating that host DNA had no adverse impact on the assay. The newly-developed RPA assay was ready to use for in-field and/or storage applications for accurate and robust detection of *Pectobacterium* sp. in tomato and potato.

Conclusion

We established a novel lateral flow strip-RPA assay for *Pectobacterium* detection that provided unique advantage with respect to speed, specificity, sensitivity, ease of visual detection and simplicity of equipment required. All these features indicated that the RPA assay can be a useful tool for laboratory and field applications for specific detection of *Pectobacterium*.

Appendix A- The antifungal activity of APF on different genera of fungal isolates

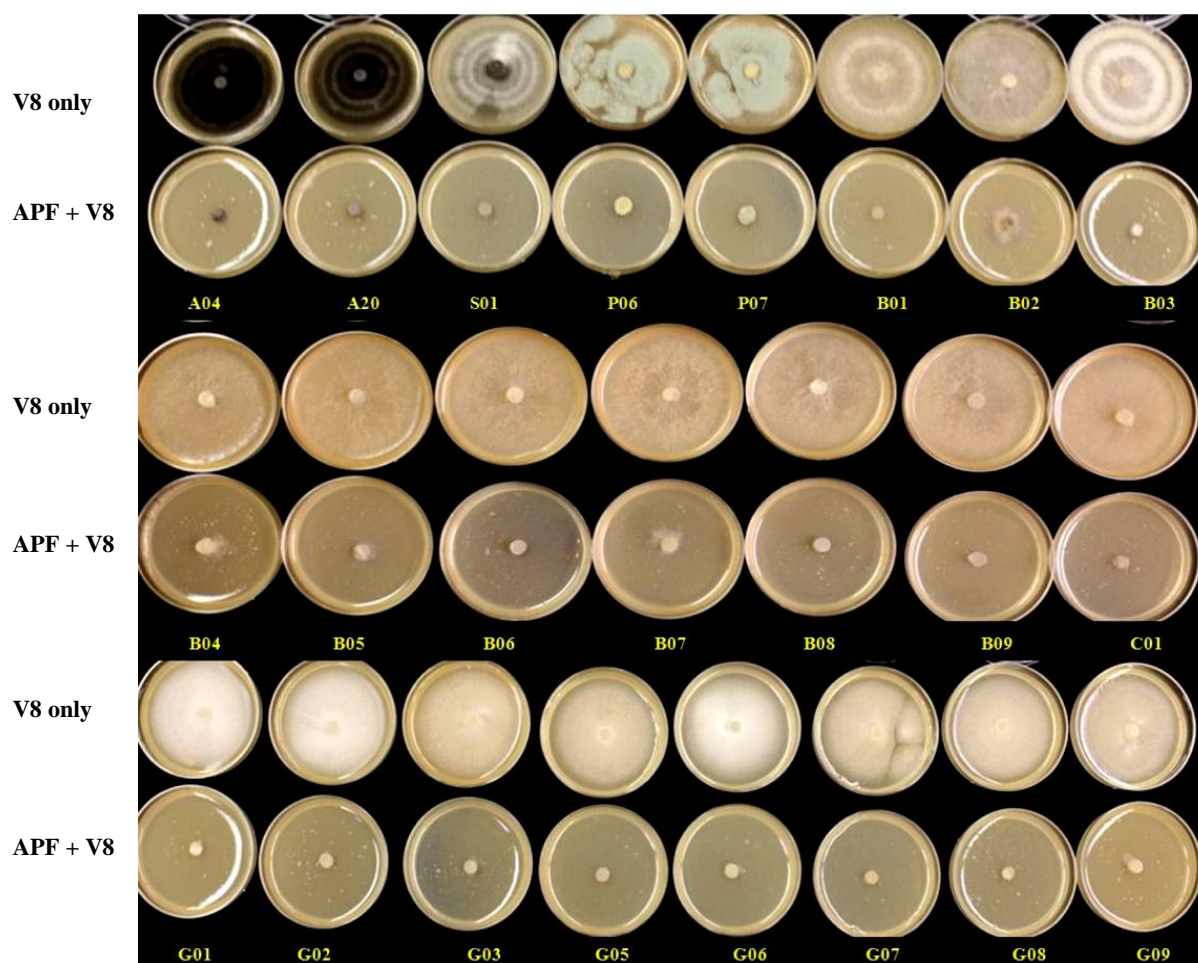


Figure A1. Mycelial growth of different fungal genera on agar containing APF at 1ml/L 72 h after incubation at 23°C using inhibitory assay. A= *Alternaria*; B= *Botrytis*; C= *Colletotrichium*; G= *Geotrichum*; P= *Penicillium*; S= *Stemphyllium*

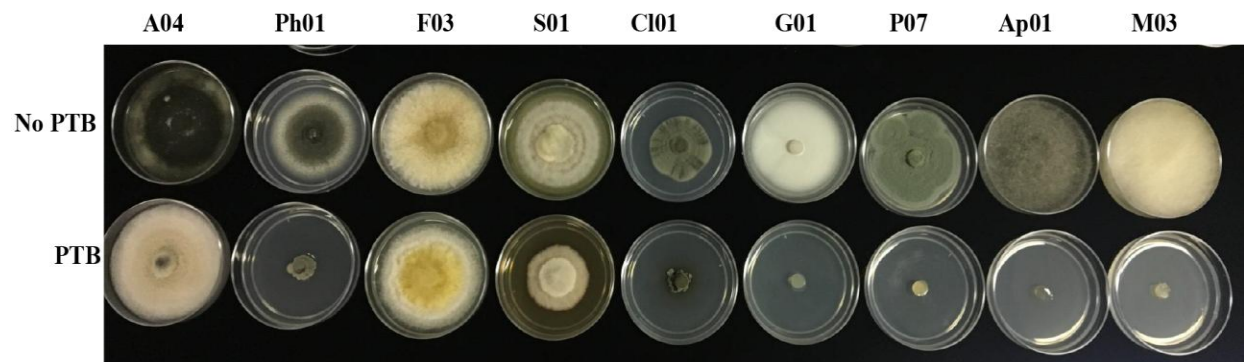


Figure A2. Mycelial growth of different fungal genera on agar containing potassium tetraborate PTB at 25 mM 72 h after incubation at 23°C using inhibitory assay. A04= *Alternaria*; Ph01= *Phoma*; F03= *Fusarium*; S= *Stemphyllium*; Cl01= *Colletotrichum* sp.; G0= *Geotrichum*; P07= *Penicillium*; Ap01= *Aspergillus* sp.; M03= *Mucor* sp.

Appendix B- The effect of crude extracts of some plants on the mycelial growth of two different genera of fungal isolates.

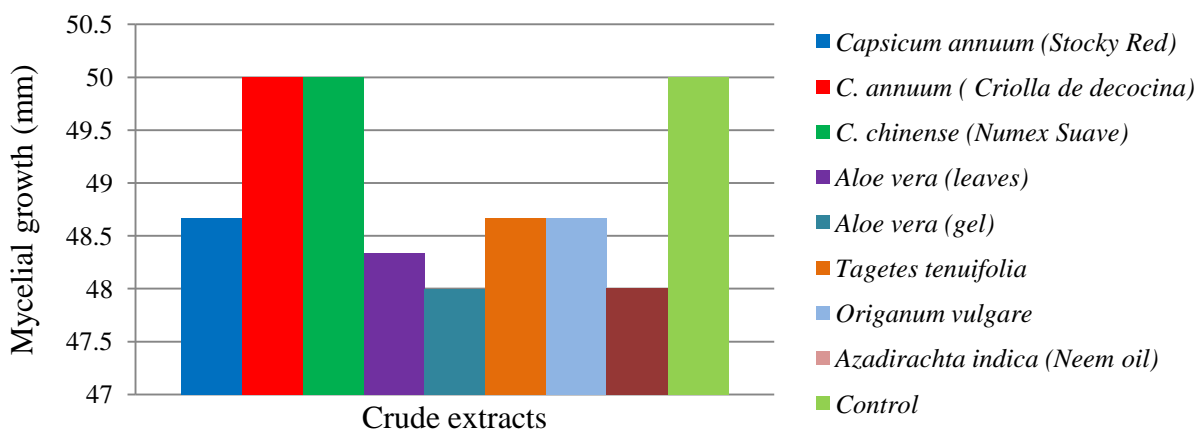


Figure B 1. The effect of crude extracts of some plants on mycelial growth of *Alternaria* sp. at 23°C.

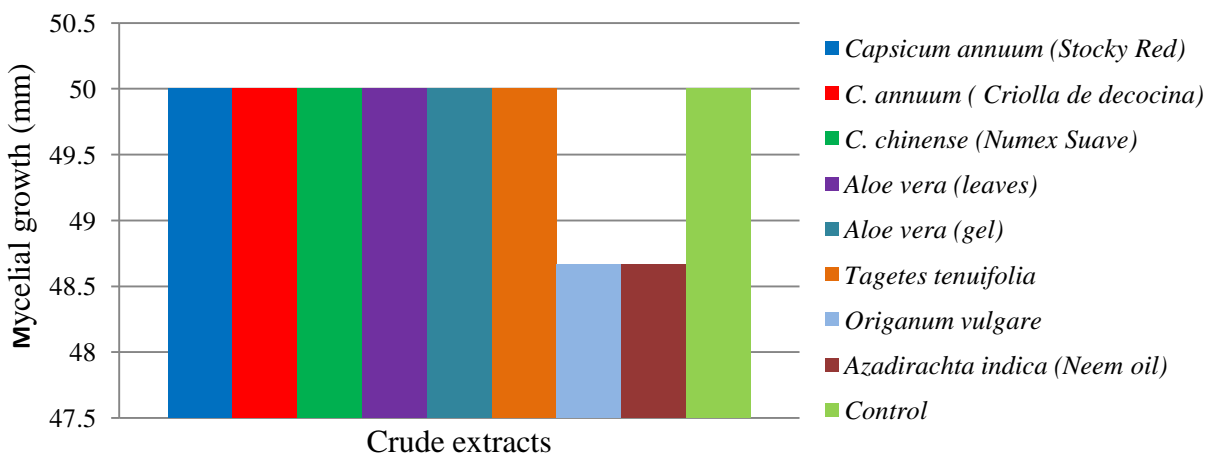


Figure B 2. The effect of crud extracts of some plants on mycelial growth of *Botrytis cinerea* at 23°C.

Appendix C- The effect of light on degradation of tested plant extracts.

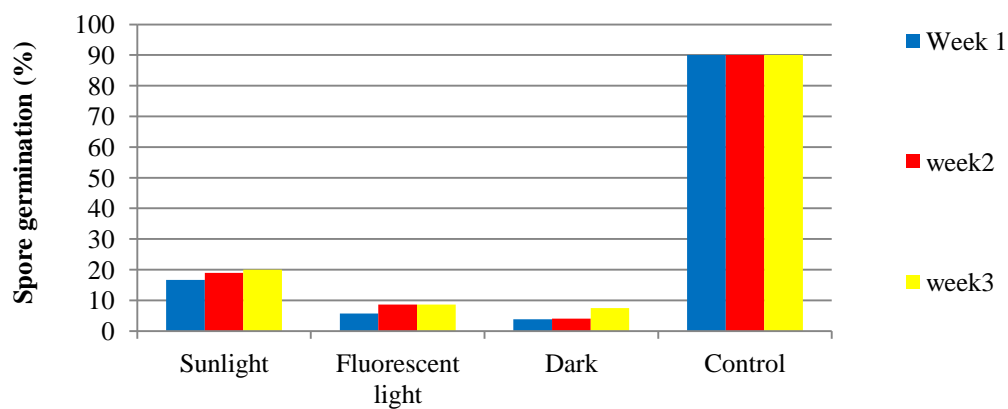


Figure C 1. The effect of light on degradation of *Capsicum chinense* extracts indicated by the percentage of spore germination of *Botrytis cinerea*

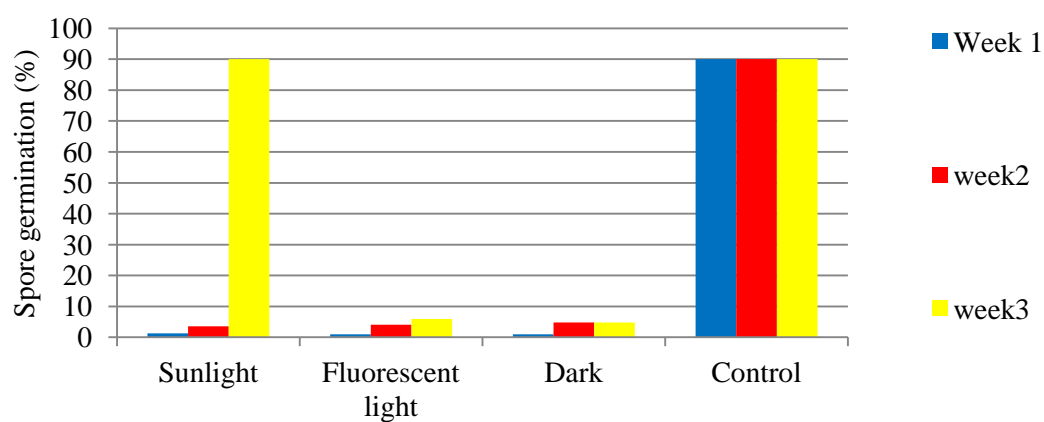


Figure C 2. The effect of light on degradation of *Capsicum annuum* extract indicated by the percentage of spore germination of *Botrytis cinerea*

Appendix D- Phytotoxicity of Agrichem proprietary formulation APF on tomato fruit and potassium tetraborate PTB on tomato plant.

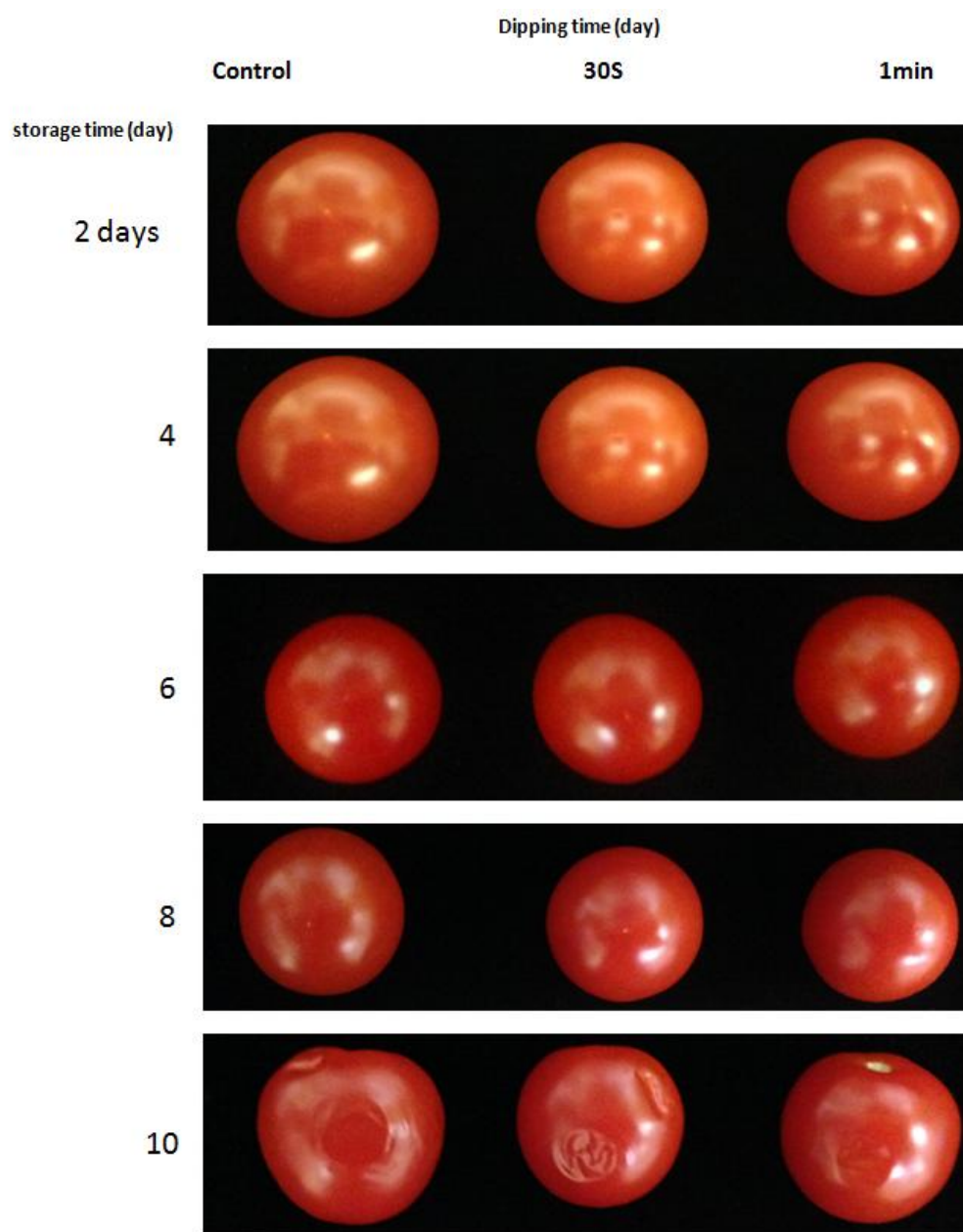


Figure D1. Phytotoxicity of APF at 1ml/L on tomato fruit with different immersion and storage time.

Table D1. The phytotoxicity of potassium tetraborate applied at different concentrations to tomato. Plants were treated at three different times of day.

Observation time	Hours after treatment						
	25	40	50	75	100	125	150
Morning	-	-	+	+	+	+	+
Afternoon	-	-	+	+	+	+	+
Night	-	-	+	+	+	+	+

+ = Phytotoxicity, -= no Phytotoxicity

Appendix E- Percentage of spore germination of *Botrytis cinerea* with different times, humidity and temperature.

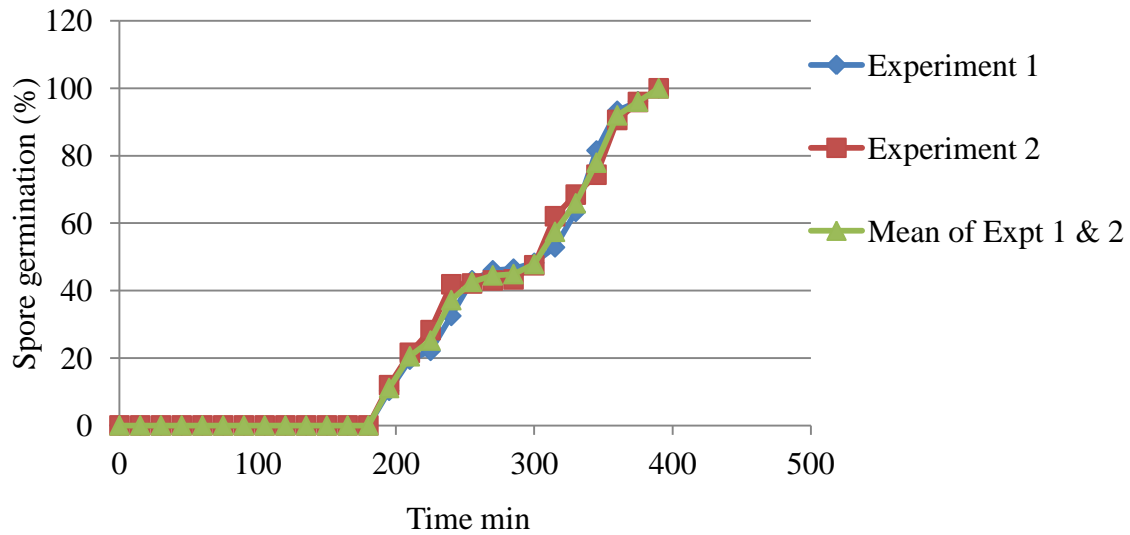


Figure E1. The percentage germination of *Botrytis cinerea* spores over time.

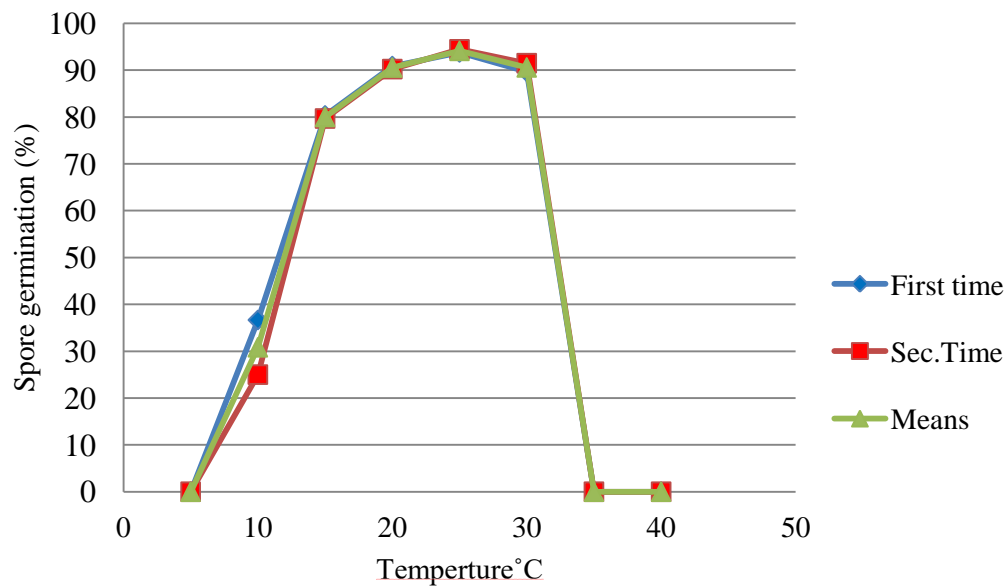


Figure E2. The percentage of spore germination of *Botrytis cinerea* with temperature

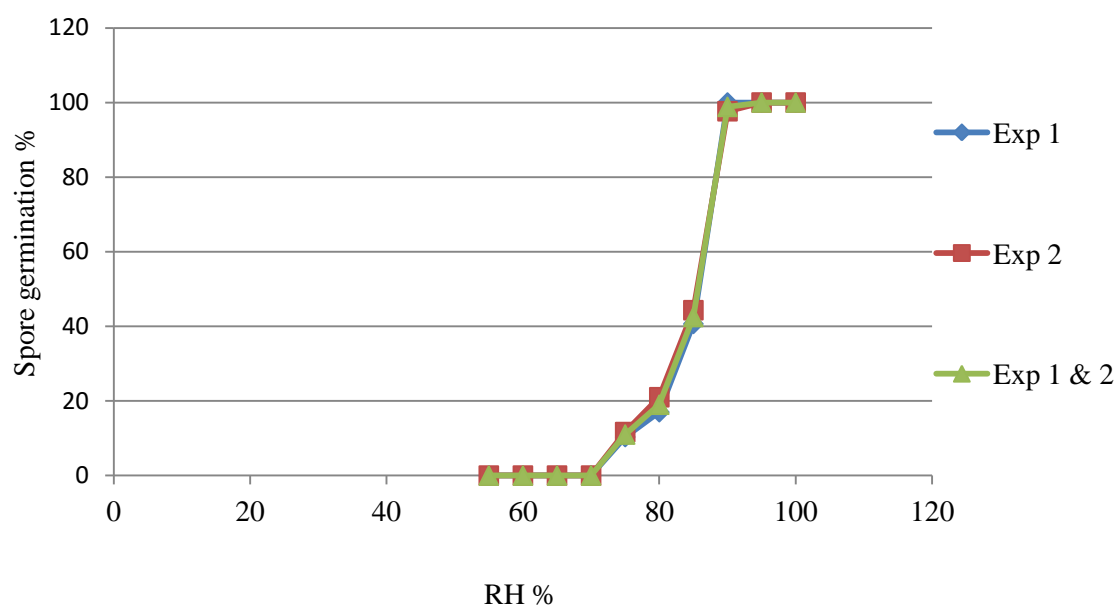


Figure E3. The percentage germination of *Botrytis cinerea* spores with different humidities

Appendix F - The efficacy of potassium tetraborate PTB on disease severity of tomato fruit using a curative assay.

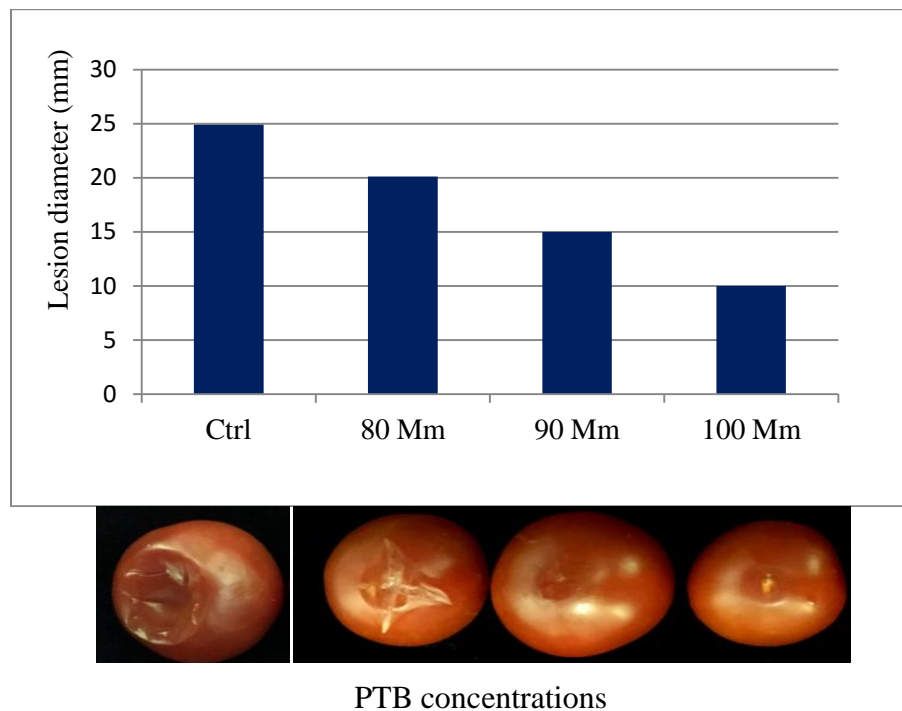


Figure F1. The efficacy of potassium tetraborate (PTB) on tomato fruit disease severity with varying concentrations and 10 min immersion time. Fruit were treated 10 min after inoculation with *Pectobacterium carotovorum*. Inoculated fruit were stored for 7 days at 28°C then evaluated.

Table F1. The efficacy of potassium tetraborate (PTB) on tomato fruit disease severity with varying concentrations at 10 min spray time. Fruit were sprayed 10 min after inoculation with *Pectobacterium carotovorum* Inoculated fruit were stored for 7 days at 28°C then evaluated.

PTB concentration (mM)	Lesion diameter (mm)
0 (water only)	Soft all
80	Soft all
90	Soft all
100	Soft all



CHAPTER VII.

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